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(54) Title: REGULATION OF HUMAN PHOSPHOLIPASE-LIKE ENZYME

(57) **Abstract:** Reagents which regulate human phospholipase-like enzyme and reagents which bind to human phospholipase-like enzyme gene products can play a role in preventing, ameliorating, or correcting dysfunctions or diseases including, but not limited to, cancer, inflammation, CNS disorders, cardiovascular disorders, chronic obstructive pulmonary disease, diabetes, and obesity.

REGULATION OF HUMAN PHOSPHOLIPASE-LIKE ENZYME

This application incorporates by reference Serial No. 60/238,445 filed October 10, 2000.

TECHNICAL FIELD OF THE INVENTION

The invention relates to the area of enzyme regulation. More particularly, the invention relates to the regulation of human phospholipase-like enzyme and its regulation.

BACKGROUND OF THE INVENTION

The secretory phospholipase A2 (PLA2) superfamily comprises a number of heterogeneous enzymes whose common feature is to hydrolyze the sn-2 fatty acid acyl ester bond of phosphoglycerides. U.S. Patent No. 6,103,469. Hydrolysis of the glycerophospholipids releases free fatty acids and lysophospholipids. PLA2 activity generates precursors for the biosynthesis of biologically active lipids, hydroxy fatty acids, and platelet-activating factor.

PLA2 hydrolysis of the sn-2 ester bond in phospholipids generates free fatty acids, such as arachidonic acid and lysophospholipids. Arachidonic acid is processed into bioactive lipid mediators of inflammation, such as lyso-platelet-activating factor and eicosanoids. The synthesis of arachidonic acid from membrane phospholipids is the rate-limiting step in the biosynthesis of the four major classes of eicosanoids (prostaglandins, prostacyclins, thromboxanes and leukotrienes) involved in pain, fever, and inflammation. Furthermore, leukotriene-B4 is known to function in a feedback loop which further increases PLA2 activity. Wijkander *et al.*, *J. Biol. Chem. 270*, 26543-49, 1995.

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PLA2s, first described as components of snake venoms and pancreatic juice and later defined in mammals, have traditionally been classified into several major groups and subgroups based on their amino acid sequence, disulfide bond location, and divalent cation requirements. The PLA2s of groups I, II, and III consist of low molecular

5   weight, secreted, Ca^{2+}-dependent proteins, Group IV PLA2s are primarily 85-kDa, Ca^{2+}-dependent cytosolic phospholipases, and an additional group comprises the Ca^{2+}-independent cytosolic phospholipases. Davidson & Dennis, *J. Mol. Evol. 31*, 228-38, 1990; Dennis, *J. Biol. Chem. 269*, 13057-13060, 1994.

10   The first PLA2s to be extensively characterized were the Group I, II, and III PLA2s found in snake and bee venoms. These venom PLA2s share many features with mammalian PLA2s, including a common catalytic mechanism, the same Ca+ requirement, and conserved primary and tertiary structures. In addition to their role in the digestion of prey, the venom PLA2s display neurotoxic, myotoxic, anti-

15   coagulant, and proinflammatory effects in mammalian tissues. This diversity of pathophysiological effects is due to the presence of specific, high affinity receptors for these enzymes on various cells and tissues. Lambeau *et al.*, *J. Biol. Chem. 270*, 5534-40, 1995.

20   At least four different groups of PLA2s have been characterized in mammalian cells, including Group I (pancreatic), Groups IIA, and IIC (inflammatory), and Group V (expressed in the heart). The pancreatic PLA2s function in the digestion of dietary lipids and have been proposed to play a role in cell proliferation, smooth muscle contraction, and acute lung injury. The inflammatory PLA2s are potent mediators of

25   inflammatory processes and are highly expressed in serum and synovial fluids of patients with inflammatory disorders. These Group II PLA2s are found in most human cell types assayed and are expressed in diverse pathological processes such as septic shock, intestinal cancers, rheumatoid arthritis, and epidermal hyperplasia. A Group V PLA2 has been cloned from brain tissue and is strongly expressed in heart

30   tissue. Other PLA2s have been cloned from various human tissues and cell lines, suggesting a large diversity of PLA2s. A human PLA2 was recently cloned from

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fetal lung, and based on its structural properties, appears to be the first member of a new group of mammalian PLAs, referred to as Group X. Chen *et al.*, *J. Biol. Chem. 269*, 2365-68, 1994; Kennedy *et al.*, *J. Biol. Chem. 270*, 22378-85, 1995; Komada *et al.*, *Biochem. Biophys. Res. Commun. 168*, 1059-65, 1990; and Cupillard *et al.*, *J.*

5   *Biol. Chem. 272*, 15745-52, 1997).

The discovery of a new human phospholipase A2 protein and the polynucleotides encoding it satisfies a need in the art by providing new compositions which are useful in the diagnosis, prevention, and treatment of cancer, inflammation, CNS

10   disorders, cardiovascular disorders, chronic obstructive pulmonary disease, diabetes, and obesity.

SUMMARY OF THE INVENTION

15   It is an object of the invention to provide reagents and methods of regulating a human phospholipase-like enzyme. This and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention is a phospholipase-like enzyme polypeptide

20   comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 77% identical to the amino acid sequence shown in SEQ ID NO: 2; and

25   the amino acid sequence shown in SEQ ID NO: 2.

Yet another embodiment of the invention is a method of screening for agents which decrease extracellular matrix degradation. A test compound is contacted with a phospholipase-like enzyme polypeptide comprising an amino acid sequence selected

30   from the group consisting of:

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amino acid sequences which are at least about 77% identical to the amino acid sequence shown in SEQ ID NO: 2; and

the amino acid sequence shown in SEQ ID NO: 2.

Binding between the test compound and the phospholipase-like enzyme polypeptide is detected. A test compound which binds to the phospholipase-like enzyme polypeptide is thereby identified as a potential agent for decreasing extracellular matrix degradation. The agent can work by decreasing the activity of the phospholipase-like enzyme.

Another embodiment of the invention is a method of screening for agents which decrease extracellular matrix degradation. A test compound is contacted with a polynucleotide encoding a phospholipase-like enzyme polypeptide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1; and

the nucleotide sequence shown in SEQ ID NO: 1.

Binding of the test compound to the polynucleotide is detected. A test compound which binds to the polynucleotide is identified as a potential agent for decreasing extracellular matrix degradation. The agent can work by decreasing the amount of the phospholipase-like enzyme through interacting with the phospholipase-like enzyme mRNA.

Another embodiment of the invention is a method of screening for agents which regulate extracellular matrix degradation. A test compound is contacted with a phospholipase-like enzyme polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 77% identical to the amino acid sequence shown in SEQ ID NO: 2; and

5   the amino acid sequence shown in SEQ ID NO: 2.

A phospholipase-like enzyme activity of the polypeptide is detected. A test compound which increases phospholipase-like enzyme activity of the polypeptide relative to phospholipase-like enzyme activity in the absence of the test compound is thereby
10   identified as a potential agent for increasing extracellular matrix degradation. A test compound which decreases phospholipase-like enzyme activity of the polypeptide relative to phospholipase-like enzyme activity in the absence of the test compound is thereby identified as a potential agent for decreasing extracellular matrix degradation.

15   Even another embodiment of the invention is a method of screening for agents which decrease extracellular matrix degradation. A test compound is contacted with a phospholipase-like enzyme product of a polynucleotide which comprises a nucleotide sequence selected from the group consisting of:

20   nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO: 1; and

the nucleotide sequence shown in SEQ ID NO: 1.

25   Binding of the test compound to the phospholipase-like enzyme product is detected. A test compound which binds to the phospholipase-like enzyme product is thereby identified as a potential agent for decreasing extracellular matrix degradation.

Still another embodiment of the invention is a method of reducing extracellular
30   matrix degradation. A cell is contacted with a reagent which specifically binds to a polynucleotide encoding a phospholipase-like enzyme polypeptide or the product

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encoded by the polynucleotide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide
5   sequence shown in SEQ ID NO: 1; and

the nucleotide sequence shown in SEQ ID NO: 1.

Phospholipase-like enzyme activity in the cell is thereby decreased.
10

The invention thus provides a human phospholipase-like enzyme which can be used to identify test compounds which may act, for example, as activators or inhibitors at the enzyme's active site. Human phospholipase-like enzyme and fragments thereof also are useful in raising specific antibodies which can block the enzyme and
15   effectively reduce its activity.

BRIEF DESCRIPTION OF THE DRAWINGS

       Fig. 1   shows the DNA-sequence encoding a phospholipase-like enzyme Poly-
20   peptide (SEQ ID NO: 1).

       Fig. 2   shows the amino acid sequence deduced from the DNA-sequence of Fig.1
                   (SEQ ID NO: 2).

25   Fig. 3   shows the amino acid sequence of the protein identified by SwissProt
                   Accession No. Q05017 (SEQ ID NO: 3).

       Fig. 4   shows the DNA-sequence encoding a phospholipase-like enzyme Poly-
                   peptide (SEQ ID NO: 4).

30

Fig. 5   shows the DNA-sequence encoding a phospholipase-like enzyme Poly-
           peptide (SEQ ID NO: 5).

Fig. 6   shows the DNA-sequence encoding a phospholipase-like enzyme Poly-
5   peptide (SEQ ID NO: 6).

Fig. 7   shows the DNA-sequence encoding a phospholipase-like enzyme Poly-
           peptide (SEQ ID NO: 7).

10   Fig. 8   shows the BLASTP alignment of human phospholipase-like enzyme (SEQ
                 ID NO: 2) with the protein identified with SwissProt Accession No.
                 Q05017 (SEQ ID NO: 3).

Fig. 9   shows the Prosite search results.

15

Fig. 10   shows the HMMPFAM alignment of SEQ ID NO: 2 with
           pfam|hmm|Lipase_GDSL.

Fig. 11   shows the HMMPFAM alignment of SEQ ID NO: 2 with
20   pfam|hmm|Lipase_GDSL.

Fig. 12   shows the HMMPFAM alignment of SEQ ID NO: 2 with
           pfam|hmm|Lipase_GDSL.

25   **DETAILED DESCRIPTION OF THE INVENTION**

The invention relates to an isolated polynucleotide encoding a phospholipase-like
enzyme polypeptide and being selected from the group consisting of:

30   a)   a polynucleotide encoding a phospholipase-like enzyme polypeptide com-
             prising an amino acid sequence selected from the group consisting of:

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amino acid sequences which are at least about 77% identical to
the amino acid sequence shown in SEQ ID NO: 2; and

the amino acid sequence shown in SEQ ID NO: 2;

5

b)   a polynucleotide comprising the sequence of SEQ ID NO: 1;

c)   a polynucleotide which hybridizes under stringent conditions to a poly-
        nucleotide specified in (a) and (b);

10

d)   a polynucleotide the sequence of which deviates from the polynucleotide
        sequences specified in (a) to (c) due to the degeneration of the genetic code;
        and

15   e)   a polynucleotide which represents a fragment, derivative or allelic variation of
        a polynucleotide sequence specified in (a) to (d).

Furthermore, it has been discovered by the present applicant that a novel
phospholipase-like enzyme, particularly a human phospholipase-like enzyme, is a
20   discovery of the present invention. Human phospholipase-like enzyme comprises the
amino acid sequence shown in SEQ ID NO: 2. A coding sequence for human
phospholipase-like enzyme is shown in SEQ ID NO: 1. This coding sequence is found in
GENBANK|AC074011.2 (genomic, chromosome 2) and GENBANK|AC022076.10 (partial
genomic sequence on chromosome 3); this gene is duplicated on chromosome 2 and 3.
25   Related ESTs (SEQ ID NOS: 4-7) are expressed in squamous cell carcinomas,
pooled human melanocyte, fetal heart, pregnant uterus, and adult colon.

Human phospholipase-like enzyme is 77% identical over 1217 amino acids to the
rabbit protein identified with SwissProt Accession No. Q05017 and annotated as
30   "PHOSPHOLIPASE ADRAB-B PRECURSOR (EC 3.1.-.-)" (Fig. 8).

By inference from sequence similarities, human phospholipase-like enzyme belongs to the "GDSL" family of lipolytic enzymes and has both esterase and phospholipase A/lysopholilipase activities. Human phospholipase-like enzyme of the invention is expected to be useful for the same purposes as previously identified phospholipase

5   enzymes. Human phospholipase-like enzyme is believed to be useful in therapeutic methods to treat disorders such as cancer, inflammation, CNS disorders, cardio-vascular disorders, chronic obstructive pulmonary disease, diabetes, and obesity. Human phospholipase-like enzyme also can be used to screen for human phospholipase-like enzyme activators and inhibitors.

10

Polypeptides

Human phospholipase-like enzyme polypeptides according to the invention comprise at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450,

15   500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, or 1210 contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO: 2or a biologically active variant thereof, as defined below. A phospholipase-like enzyme polypeptide of the invention therefore can be a portion of a phospholipase-like enzyme protein, a full-length phospholipase-like enzyme protein,

20   or a fusion protein comprising all or a portion of a phospholipase-like enzyme protein.

Biologically Active Variants

25   Human phospholipase-like enzyme polypeptide variants which are biologically active, _e.g._, retain a phospholipase or esterase activity, also are phospholipase-like enzyme polypeptides. Preferably, naturally or non-naturally occurring phospholipase-like enzyme polypeptide variants have amino acid sequences which are at least about 77, 80, 85, 90, 96, 96, or 98% identical to the amino acid sequence shown in SEQ ID

30   NO: 2 or a fragment thereof. Percent identity between a putative phospholipase-like enzyme polypeptide variant and an amino acid sequence of SEQ ID NO: 2 is

determined using the Blast2 alignment program (Blosum62, Expect 10, standard genetic codes).

5   Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

10

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity of a phospholipase-like enzyme polypeptide can

15   be found using computer programs well known in the art, such as DNASTAR software. Whether an amino acid change results in a biologically active phospholipase-like enzyme polypeptide can readily be determined by assaying for phospholipase activity, as described for example, in Example 4.

20   *Fusion Proteins*

Fusion proteins are useful for generating antibodies against phospholipase-like enzyme polypeptide amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins which interact with portions

25   of a phospholipase-like enzyme polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

30   A phospholipase-like enzyme polypeptide fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment

comprises at least 6, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, or 1210 contiguous amino acids of SEQ ID NO: 2 or of a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length phospholipase-like enzyme protein.

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include β-galactosidase, β-glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horse-radish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Additionally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the phospholipase-like enzyme polypeptide-encoding sequence and the heterologous protein sequence, so that the phospholipase-like enzyme polypeptide can be cleaved and purified away from the heterologous moiety.

A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can be used to prepare fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from the complement of SEQ ID NO: 1 in proper reading frame with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International

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Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

Identification of Species Homologs

Species homologs of human phospholipase-like enzyme polypeptide can be obtained using phospholipase-like enzyme polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of phospholipase-like enzyme polypeptide, and expressing the cDNAs as is known in the art.

Polynucleotides

A phospholipase-like enzyme polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for a phospholipase-like enzyme polypeptide. A partial coding sequence for human phospholipase-like enzyme is shown in SEQ ID NO: 1.

Degenerate nucleotide sequences encoding human phospholipase-like enzyme polypeptides, as well as homologous nucleotide sequences which are at least about 50, 55, 60, 65, 70, preferably about 75, 90, 96, or 98% identical to the nucleotide sequence shown in SEQ ID NO: 1 or its complement also are phospholipase-like enzyme polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of phospholipase-like enzyme polynucleotides which encode biologically active phospholipase-like enzyme polypeptides also are phospholipase-like enzyme polynucleotides. Polynucleotides comprising at least 6, 7, 8, 9, 10, 12, 15, 18, 20, or 25 contiguous nucleotides of SEQ ID NO: 1 or its

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complement also are human phospholipase-like enzyme polynucleotides. Such poly-nucleotides can be used, for example, as hybridization probes or antisense oligo-nucleotides.

5   *Identification of Polynucleotide Variants and Homologs*

Variants and homologs of the phospholipase-like enzyme polynucleotides described above also are phospholipase-like enzyme polynucleotides. Typically, homologous phospholipase-like enzyme polynucleotide sequences can be identified by hybri-

10   dization of candidate polynucleotides to known phospholipase-like enzyme polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50°C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes

15   each--homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the phospholipase-like enzyme polynucleotides disclosed herein

20   also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of phospholipase-like enzyme polynucleotides can be identified, for example, by screening human cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5°C with every 1% decrease in

25   homology (Bonner *et al.*, *J. Mol. Biol. 81*, 123 (1973). Variants of human phospholipase-like enzyme polynucleotides or phospholipase-like enzyme poly-nucleotides of other species can therefore be identified by hybridizing a putative homologous phospholipase-like enzyme polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO: 1 or the complement thereof to form a test

30   hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary

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nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to phospholipase-like enzyme polynucleotides or their complements following stringent hybridization and/or wash conditions also are phospholipase-like enzyme polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20°C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a phospholipase-like enzyme polynucleotide having a nucleotide sequence shown in SEQ ID NO: 1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A. 48*, 1390 (1962):

$$T_m = 81.5\ ^\circ C - 16.6(\log_{10}[Na^+]) + 0.41(\%G + C) - 0.63(\%formamide) - 600/l,$$

where l = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65°C, or 50% formamide, 4X SSC at 42°C, or 0.5X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions include, for example, 0.2X SSC at 65°C.

Preparation of Polynucleotides

A phospholipase-like enzyme polynucleotide can be isolated free of other cellular components such as membrane components, proteins, and lipids. Polynucleotides can be made by a cell and isolated using standard nucleic acid purification techniques, or synthesized using an amplification technique, such as the polymerase

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chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating polynucleotides are routine and are known in the art. Any such technique for obtaining a polynucleotide can be used to obtain isolated phospholipase-like enzyme polynucleotides. For example, restriction enzymes and probes can be used to isolate
5   polynucleotide fragments which comprises phospholipase-like nucleotide sequences. Isolated polynucleotides are in preparations which are free or at least 70, 80, or 90% free of other molecules.

Human phospholipase-like enzyme cDNA molecules can be made with standard
10   molecular biology techniques, using phospholipase-like enzyme mRNA as a template. Human phospholipase-like enzyme cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.* (1989). An amplification technique, such as PCR, can be used to obtain additional copies of polynucleotides of the invention, using
15   either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesizes phospholipase-like enzyme polynucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a
20   phospholipase-like enzyme polypeptide having, for example, an amino acid sequence shown in SEQ ID NO: 2 or a biologically active variant thereof.

Extending Polynucleotides

25   The partial sequence disclosed herein can be used to identify the corresponding full length gene from which it was derived. The partial sequences can be nick-translated or end-labeled with ^{32}P using polynucleotide kinase using labeling methods known to those with skill in the art (BASIC METHODS IN MOLECULAR BIOLOGY, Davis *et al.*, eds., Elsevier Press, N.Y., 1986). A lambda library prepared from human tissue can
30   be directly screened with the labeled sequences of interest or the library can be converted en masse to pBluescript (Stratagene Cloning Systems, La Jolla, Calif.

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92037) to facilitate bacterial colony screening (see Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press (1989, pg. 1.20).

5   Both methods are well known in the art. Briefly, filters with bacterial colonies containing the library in pBluescript or bacterial lawns containing lambda plaques are denatured, and the DNA is fixed to the filters. The filters are hybridized with the labeled probe using hybridization conditions described by Davis *et al.*, 1986. The partial sequences, cloned into lambda or pBluescript, can be used as positive controls

10   to assess background binding and to adjust the hybridization and washing stringencies necessary for accurate clone identification. The resulting autoradio-grams are compared to duplicate plates of colonies or plaques; each exposed spot corresponds to a positive colony or plaque. The colonies or plaques are selected, expanded and the DNA is isolated from the colonies for further analysis and

15   sequencing.

Positive cDNA clones are analyzed to determine the amount of additional sequence they contain using PCR with one primer from the partial sequence and the other primer from the vector. Clones with a larger vector-insert PCR product than the

20   original partial sequence are analyzed by restriction digestion and DNA sequencing to determine whether they contain an insert of the same size or similar as the mRNA size determined from Northern blot Analysis.

Once one or more overlapping cDNA clones are identified, the complete sequence of

25   the clones can be determined, for example after exonuclease III digestion (McCombie *et al.*, *Methods 3*, 33-40, 1991). A series of deletion clones are generated, each of which is sequenced. The resulting overlapping sequences are assembled into a single contiguous sequence of high redundancy (usually three to five overlapping sequences at each nucleotide position), resulting in a highly accurate

30   final sequence.

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Various PCR-based methods can be used to extend the nucleic acid sequences disclosed herein to detect upstream sequences such as promoters and regulatory elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, *PCR Methods Applic. 2*, 318-322, 1993). Genomic DNA is first amplified in the presence of a primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR also can be used to amplify or extend sequences using divergent primers based on a known region (Triglia *et al.*, *Nucleic Acids Res. 16*, 8186, 1988). Primers can be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, Minn.), to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68-72°C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which can be used is capture PCR, which involves PCR amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom *et al.*, *PCR Methods Applic. 1*, 111-119, 1991). In this method, multiple restriction enzyme digestions and ligations also can be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

Another method which can be used to retrieve unknown sequences is that of Parker *et al.*, *Nucleic Acids Res. 19*, 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to

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walk genomic DNA (CLONTECH, Palo Alto, Calif.). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Randomly-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries can be useful for extension of sequence into 5' non-transcribed regulatory regions.

Commercially available capillary electrophoresis systems can be used to analyze the size or confirm the nucleotide sequence of PCR or sequencing products. For example, capillary sequencing can employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity can be converted to electrical signal using appropriate software (e.g. GENOTYPER and Sequence NAVIGATOR, Perkin Elmer), and the entire process from loading of samples to computer analysis and electronic data display can be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

Obtaining Polypeptides

Human phospholipase-like enzyme polypeptides can be obtained, for example, by purification from human cells, by expression of phospholipase-like enzyme poly-nucleotides, or by direct chemical synthesis.

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Protein Purification

Human phospholipase-like enzyme polypeptides can be purified from any cell which
expresses the enzyme, including host cells which have been transfected with
phospholipase-like enzyme expression constructs. A purified phospholipase-like
enzyme polypeptide is separated from other compounds which normally associate
with the phospholipase-like enzyme polypeptide in the cell, such as certain proteins,
carbohydrates, or lipids, using methods well-known in the art. Such methods
include, but are not limited to, size exclusion chromatography, ammonium sulfate
fractionation, ion exchange chromatography, affinity chromatography, and prepa-
rative gel electrophoresis. A preparation of purified phospholipase-like enzyme
polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99%
pure. Purity of the preparations can be assessed by any means known in the art, such
as SDS-polyacrylamide gel electrophoresis.

Expression of Polynucleotides

To express a phospholipase-like enzyme polynucleotide, the polynucleotide can be
inserted into an expression vector which contains the necessary elements for the
transcription and translation of the inserted coding sequence. Methods which are
well known to those skilled in the art can be used to construct expression vectors
containing sequences encoding phospholipase-like enzyme polypeptides and appro-
priate transcriptional and translational control elements. These methods include *in
vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic
recombination. Such techniques are described, for example, in Sambrook *et al.*
(1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John
Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be utilized to contain and express
sequences encoding a phospholipase-like enzyme polypeptide. These include, but

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are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (e.g., baculovirus), plant cell systems transformed with virus expression vectors (e.g.,

5   cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those non-translated regions of the vector -- enhancers, promoters, 5' and 3' untranslated regions -- which interact with

10 host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the

15 BLUESCRIPT phagemid (Stratagene, LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) can be cloned into the vector. In mammalian

20 cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding a phospholipase-like enzyme polypeptide, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

25 _Bacterial and Yeast Expression Systems_

In bacterial systems, a number of expression vectors can be selected depending upon the use intended for the phospholipase-like enzyme polypeptide. For example, when a large quantity of a phospholipase-like enzyme polypeptide is needed for the

30 induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified can be used. Such vectors include, but are not limited to,

multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene). In a BLUESCRIPT vector, a sequence encoding the phospholipase-like enzyme polypeptide can be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β-galactosidase so that a hybrid protein is produced. pIN vectors (Van Heeke & Schuster, *J. Biol. Chem. 264*, 5503-5509, 1989) or pGEX vectors (Promega, Madison, Wis.) also can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems can be designed to include heparin, thrombin, or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH can be used. For reviews, see Ausubel *et al.* (1989) and Grant *et al., Methods Enzymol. 153*, 516-544, 1987.

Plant and Insect Expression Systems

If plant expression vectors are used, the expression of sequences encoding phospholipase-like enzyme polypeptides can be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV can be used alone or in combination with the omega leader sequence from TMV (Takamatsu, *EMBO J. 6*, 307-311, 1987). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters can be used (Coruzzi *et al., EMBO J. 3*, 1671-1680, 1984; Broglie *et al., Science 224*, 838-843, 1984; Winter *et al., Results Probl. Cell Differ. 17*, 85-105, 1991). These constructs can be introduced into plant cells by direct DNA transformation or by pathogen-mediated transfection. Such techniques are described in a number of generally available

reviews (*e.g.*, Hobbs or Murray, in MCGRAW HILL YEARBOOK OF SCIENCE AND TECHNOLOGY, McGraw Hill, New York, N.Y., pp. 191-196, 1992).

An insect system also can be used to express a phospholipase-like enzyme polypeptide. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. Sequences encoding phospholipase-like enzyme polypeptides can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of phospholipase-like enzyme polypeptides will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses can then be used to infect *S. frugiperda cells* or *Trichoplusia* larvae in which phospholipase-like enzyme polypeptides can be expressed (Engelhard *et al.*, *Proc. Nat. Acad. Sci. 91*, 3224-3227, 1994).

Mammalian Expression Systems

A number of viral-based expression systems can be used to express phospholipase-like enzyme polypeptides in mammalian host cells. For example, if an adenovirus is used as an expression vector, sequences encoding phospholipase-like enzyme polypeptides can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing a phospholipase-like enzyme polypeptide in infected host cells (Logan & Shenk, *Proc. Natl. Acad. Sci. 81*, 3655-3659, 1984). If desired, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, can be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) also can be used to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6M to 10M are

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constructed and delivered to cells via conventional delivery methods (*e.g.*, liposomes, polycationic amino polymers, or vesicles).

Specific initiation signals also can be used to achieve more efficient translation of sequences encoding phospholipase-like enzyme polypeptides. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding a phospholipase-like enzyme polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals (including the ATG initiation codon) should be provided. The initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used (see Scharf *et al.*, *Results Probl. Cell Differ.* 20, 125-162, 1994).

Host Cells

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed phospholipase-like enzyme polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (*e.g.*, CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

Stable expression is preferred for long-term, high-yield production of recombinant proteins. For example, cell lines which stably express phospholipase-like enzyme polypeptides can be transformed using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced phospholipase-like enzyme sequences. Resistant clones of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. See, for example, ANIMAL CELL CULTURE, R.I. Freshney, ed., 1986.

Any number of selection systems can be used to recover transformed cell lines.

These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, *Cell 11*, 223-32, 1977) and adenine phosphoribosyltransferase (Lowy *et al.*, *Cell 22*, 817-23, 1980) genes which can be employed in *tk⁻* or *aprt⁻* cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate (Wigler *et al.*, *Proc. Natl. Acad. Sci. 77*, 3567-70, 1980), *npt* confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin *et al.*, *J. Mol. Biol. 150*, 1-14, 1981), and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murray, 1992, *supra*). Additional selectable genes have been described. For example, *trpB* allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci. 85*, 8047-51, 1988). Visible markers such as anthocyanins, β-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, can be used to identify transformants and to quantify the amount

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of transient or stable protein expression attributable to a specific vector system (Rhodes *et al.*, *Methods Mol. Biol. 55*, 121-131, 1995).

Detecting Expression

Although the presence of marker gene expression suggests that the phospholipase-like enzyme polynucleotide is also present, its presence and expression may need to be confirmed. For example, if a sequence encoding a phospholipase-like enzyme polypeptide is inserted within a marker gene sequence, transformed cells containing sequences which encode a phospholipase-like enzyme polypeptide can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding a phospholipase-like enzyme polypeptide under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the phospholipase-like enzyme polynucleotide.

Alternatively, host cells which contain a phospholipase-like enzyme polynucleotide and which express a phospholipase-like enzyme polypeptide can be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or protein. For example, the presence of a polynucleotide sequence encoding a phospholipase-like enzyme polypeptide can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding a phospholipase-like enzyme polypeptide. Nucleic acid amplification-based assays involve the use of oligonucleotides selected from sequences encoding a phospholipase-like enzyme polypeptide to detect transformants which contain a phospholipase-like enzyme polynucleotide.

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A variety of protocols for detecting and measuring the expression of a phospholipase-like enzyme polypeptide, using either polyclonal or monoclonal antibodies specific for the polypeptide, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and

5   fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on a phospholipase-like enzyme polypeptide can be used, or a competitive binding assay can be employed. These and other assays are described in Hampton *et al.*, SEROLOGICAL METHODS: A LABORATORY MANUAL, APS Press, St. Paul, Minn.,

10   1990) and Maddox *et al.*, *J. Exp. Med. 158*, 1211-1216, 1983).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to

15   polynucleotides encoding phospholipase-like enzyme polypeptides include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a phospholipase-like enzyme polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to

20   synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent,

25   or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and Purification of Polypeptides

30   Host cells transformed with nucleotide sequences encoding a phospholipase-like enzyme polypeptide can be cultured under conditions suitable for the expression and

recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode phospholipase-like enzyme polypeptides

5   can be designed to contain signal sequences which direct secretion of soluble phospholipase-like enzyme polypeptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound phospholipase-like enzyme polypeptide.

10   As discussed above, other constructions can be used to join a sequence encoding a phospholipase-like enzyme polypeptide to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized

15   metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). Inclusion of cleavable linker sequences such as those specific for Factor Xa or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the phospholipase-like enzyme polypeptide also can be used

20   to facilitate purification. One such expression vector provides for expression of a fusion protein containing a phospholipase-like enzyme polypeptide and 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification by IMAC (immobilized metal ion affinity chromatography, as described in Porath et al., Prot. Exp. Purif. 3, 263-281, 1992), while the

25   enterokinase cleavage site provides a means for purifying the phospholipase-like enzyme polypeptide from the fusion protein. Vectors which contain fusion proteins are disclosed in Kroll et al., DNA Cell Biol. 12, 441-453, 1993.

Chemical Synthesis

Sequences encoding a phospholipase-like enzyme polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al. Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a phospholipase-like enzyme polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc. 85*, 2149-2154, 1963; Roberge *et al.*, *Science 269*, 202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of phospholipase-like enzyme polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule.

The newly synthesized peptide can be substantially purified by preparative high performance liquid chromatography (*e.g.*, Creighton, PROTEINS: STRUCTURES AND MOLECULAR PRINCIPLES, WH Freeman and Co., New York, N.Y., 1983). The composition of a synthetic phospholipase-like enzyme polypeptide can be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; *see* Creighton, *supra*). Additionally, any portion of the amino acid sequence of the phospholipase-like enzyme polypeptide can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion protein.

Production of Altered Polypeptides

As will be understood by those of skill in the art, it may be advantageous to produce phospholipase-like enzyme polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein

expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

5   The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter phospholipase-like enzyme polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene

10   fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

15   _Antibodies_

Any type of antibody known in the art can be generated to bind specifically to an epitope of a phospholipase-like enzyme polypeptide. "Antibody" as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab,

20   F(ab')$_2$, and Fv, which are capable of binding an epitope of a phospholipase-like enzyme polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, _e.g._, at least 15, 25, or 50 amino acids.

25   An antibody which specifically binds to an epitope of a phospholipase-like enzyme polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Various immunoassays can be used to identify antibodies having the desired specificity.

30   Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex

formation between an immunogen and an antibody which specifically binds to the immunogen.

Typically, an antibody which specifically binds to a phospholipase-like enzyme polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay.   Preferably, antibodies which specifically bind to phospholipase-like polypeptides do not detect other proteins in immunochemical assays and can immuno-precipitate a phospholipase-like enzyme polypeptide from solution.

Human phospholipase-like enzyme polypeptides can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal antibodies.   If desired, a phospholipase-like enzyme polypeptide can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels (*e.g.*, aluminum hydroxide), and surface active substances (*e.g.* lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). Among adjuvants used in humans, BCG (*bacilli Calmette-Guerin*) and *Corynebacterium parvum* are especially useful.

Monoclonal antibodies which specifically bind to a phospholipase-like enzyme polypeptide can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture.   These techniques include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler *et al.*, *Nature 256*, 495-497, 1985; Kozbor *et al.*, *J. Immunol. Methods 81*, 31-42, 1985; Cote *et al.*, *Proc. Natl. Acad. Sci. 80*, 2026-2030, 1983; Cole *et al.*, *Mol. Cell Biol. 62*, 109-120, 1984).

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In addition, techniques developed for the production of "chimeric antibodies," the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used (Morrison *et al.*, *Proc. Natl. Acad. Sci. 81*, 6851-6855, 1984; Neuberger *et al.*, *Nature 312*, 604-608,

5   1984; Takeda *et al.*, *Nature 314*, 452-454, 1985). Monoclonal and other antibodies also can be "humanized" to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between rodent

10   antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual residues or by grating of entire complementarity determining regions. Alternatively, humanized antibodies can be produced using recombinant methods, as described in GB2188638B. Antibodies which specifically bind to a phospholipase-like enzyme

15   polypeptide can contain antigen binding sites which are either partially or fully humanized, as disclosed in U.S. 5,565,332.

Alternatively, techniques described for the production of single chain antibodies can be adapted using methods known in the art to produce single chain antibodies which

20   specifically bind to phospholipase-like enzyme polypeptides. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobin libraries (Burton, *Proc. Natl. Acad. Sci. 88*, 11120-23, 1991).

25   Single-chain antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template (Thirion *et al.*, 1996, *Eur. J. Cancer Prev. 5*, 507-11). Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught, for example, in Coloma & Morrison, 1997, *Nat. Biotechnol. 15*,

30   159-63. Construction of bivalent, bispecific single-chain antibodies is taught in Mallender & Voss, 1994, *J. Biol. Chem. 269*, 199-206.

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A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding sequence, as described below.   Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology (Verhaar *et al.*, 1995, *Int. J. Cancer 61*, 497-501; Nicholls *et al.*, 1993, *J. Immunol. Meth. 165*, 81-91).

Antibodies which specifically bind to phospholipase-like enzyme polypeptides also can be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi *et al.*, *Proc. Natl. Acad. Sci. 86*, 3833-3837, 1989; Winter *et al.*, *Nature 349*, 293-299, 1991).

Other types of antibodies can be constructed and used therapeutically in methods of the invention. For example, chimeric antibodies can be constructed as disclosed in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO 94/13804, also can be prepared.

Antibodies according to the invention can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to which a phospholipase-like enzyme polypeptide is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

Antisense Oligonucleotides

Antisense oligonucleotides are nucleotide sequences which are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes

and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of phospholipase-like enzyme gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. *See* Brown, *Meth. Mol. Biol. 20*, 1-8, 1994; Sonveaux, *Meth. Mol. Biol. 26*, 1-72, 1994; Uhlmann *et al.*, *Chem. Rev. 90*, 543-583, 1990.

Modifications of phospholipase-like enzyme gene expression can be obtained by designing antisense oligonucleotides which will form duplexes to the control, 5', or regulatory regions of the phospholipase-like enzyme gene. Oligonucleotides derived from the transcription initiation site, *e.g.*, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Precise complementarity is not required for successful complex formation between an antisense oligonucleotide and the complementary sequence of a phospholipase-like enzyme polynucleotide. Antisense oligonucleotides which comprise, for example, 2, 3, 4, or 5 or more stretches of contiguous nucleotides which are precisely complementary to a phospholipase-like enzyme polynucleotide, each separated by a stretch of contiguous nucleotides which are not complementary to adjacent phospholipase-like enzyme nucleotides, can provide sufficient targeting specificity for phospholipase-like enzyme mRNA. Preferably, each stretch of complementary contiguous nucleotides is at least 4, 5, 6, 7, or 8 or more nucleotides in length. Non-complementary intervening sequences are preferably 1, 2, 3, or 4 nucleotides in length. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular phospholipase-like enzyme polynucleotide sequence.

Antisense oligonucleotides can be modified without affecting their ability to hybridize to a phospholipase-like enzyme polynucleotide. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, also can be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art. *See, e.g.*, Agrawal *et al.*, *Trends Biotechnol. 10*, 152-158, 1992; Uhlmann *et al.*, *Chem. Rev. 90*, 543-584, 1990; Uhlmann *et al.*, *Tetrahedron. Lett. 215*, 3539-3542, 1987.

Ribozymes

Ribozymes are RNA molecules with catalytic activity. *See, e.g.,* Cech, *Science 236,* 1532-1539; 1987; Cech, *Ann. Rev. Biochem. 59,* 543-568; 1990, Cech, *Curr. Opin. Struct. Biol. 2,* 605-609; 1992, Couture & Stinchcomb, *Trends Genet. 12,* 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (*e.g.,* Haseloff *et al.,* U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a phospholipase-like enzyme polynucleotide can be used to generate ribozymes which will specifically bind to mRNA transcribed from the phospholipase-like enzyme polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (*see* Haseloff *et al. Nature 334,* 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.,* EP 321,201).

Specific ribozyme cleavage sites within a phospholipase-like enzyme RNA target can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage site can be evaluated for secondary structural features which may render the target inoperable. Suitability of candidate phospholipase-like enzyme RNA targets also can be evaluated by testing

accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing

5 to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or

10 calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease phospholipase-like enzyme expression. Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. A

15 ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells.

As taught in Haseloff et al., U.S. Patent 5,641,673, ribozymes can be engineered so

20 that ribozyme expression will occur in response to factors which induce expression of a target gene. Ribozymes also can be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

25 *Differentially Expressed Genes*

Described herein are methods for the identification of genes whose products interact with human phospholipase-like enzyme. Such genes may represent genes which are differentially expressed in disorders including, but not limited to, cancer, inflam-

30 mation, CNS disorders, cardiovascular disorders, chronic obstructive pulmonary disease, diabetes, and obesity. Further, such genes may represent genes which are

differentially regulated in response to manipulations relevant to the progression or treatment of such diseases. Additionally, such genes may have a temporally modulated expression, increased or decreased at different stages of tissue or organism development. A differentially expressed gene may also have its expression modulated under control versus experimental conditions. In addition, the human phospholipase-like enzyme gene or gene product may itself be tested for differential expression.

The degree to which expression differs in a normal versus a diseased state need only be large enough to be visualized via standard characterization techniques such as differential display techniques. Other such standard characterization techniques by which expression differences may be visualized include but are not limited to, quantitative RT (reverse transcriptase), PCR, and Northern analysis.

Identification of Differentially Expressed Genes

To identify differentially expressed genes total RNA or, preferably, mRNA is isolated from tissues of interest. For example, RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique which does not select against the isolation of mRNA may be utilized for the purification of such RNA samples. See, for example, Ausubel _et al._, ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc. New York, 1987-1993. Large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, U.S. Patent 4,843,155.

Transcripts within the collected RNA samples which represent RNA produced by differentially expressed genes are identified by methods well known to those of skill in the art. They include, for example, differential screening (Tedder _et al._, _Proc. Natl. Acad. Sci. U.S.A. 85_, 208-12, 1988), subtractive hybridization (Hedrick _et al._, _Nature 308_, 149-53; Lee _et al._, _Proc. Natl. Acad. Sci. U.S.A. 88_, 2825, 1984),

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differential display (Liang & Pardee, *Science* 257, 967-71, 1992; U.S. Patent 5,262,311), and microarrays.

The differential expression information may itself suggest relevant methods for the treatment of disorders involving the human phospholipase-like enzyme. For example, treatment may include a modulation of expression of the differentially expressed genes and/or the gene encoding the human phospholipase-like enzyme. The differential expression information may indicate whether the expression or activity of the differentially expressed gene or gene product or the human phospholipase-like enzyme gene or gene product are up-regulated or down-regulated.

Screening Methods

The invention provides assays for screening test compounds which bind to or modulate the activity of a phospholipase-like enzyme polypeptide or a phospholipase-like enzyme polynucleotide. A test compound preferably binds to a phospholipase-like enzyme polypeptide or polynucleotide. More preferably, a test compound decreases or increases phospholipase-like by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

Test Compounds

Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and

synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. *See* Lam, *Anticancer Drug Des. 12*, 145, 1997.

5

Methods for the synthesis of molecular libraries are well known in the art (*see*, for example, DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6909, 1993; Erb *et al.* *Proc. Natl. Acad. Sci. U.S.A. 91*, 11422, 1994; Zuckermann *et al.*, *J. Med. Chem. 37*, 2678, 1994; Cho *et al.*, *Science 261*, 1303, 1993; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.*

10   *33*, 2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl. 33*, 2061; Gallop *et al.*, *J. Med. Chem. 37*, 1233, 1994). Libraries of compounds can be presented in solution (*see*, *e.g.*, Houghten, *BioTechniques 13*, 412-421, 1992), or on beads (Lam, *Nature 354*, 82-84, 1991), chips (Fodor, *Nature 364*, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*

15   *89*, 1865-1869, 1992), or phage (Scott & Smith, *Science 249*, 386-390, 1990; Devlin, *Science 249*, 404-406, 1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci. 97*, 6378-6382, 1990; Felici, *J. Mol. Biol. 222*, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

High Throughput Screening

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Test compounds can be screened for the ability to bind to phospholipase-like enzyme polypeptides or polynucleotides or to affect phospholipase-like enzyme activity or phospholipase-like enzyme gene expression using high throughput screening. Using high throughput screening, many discrete compounds can be tested in parallel so that

25   large numbers of test compounds can be quickly screened. The most widely established techniques utilize 96-well microtiter plates. The wells of the microtiter plates typically require assay volumes that range from 50 to 500 µl. In addition to the plates, many instruments, materials, pipettors, robotics, plate washers, and plate readers are commercially available to fit the 96-well format.

30

Alternatively, "free format assays," or assays that have no physical barrier between samples, can be used. For example, an assay using pigment cells (melanocytes) in a simple homogeneous assay for combinatorial peptide libraries is described by Jayawickreme *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 19,* 1614-18 (1994). The cells are
5   placed under agarose in petri dishes, then beads that carry combinatorial compounds are placed on the surface of the agarose. The combinatorial compounds are partially released the compounds from the beads. Active compounds can be visualized as dark pigment areas because, as the compounds diffuse locally into the gel matrix, the active compounds cause the cells to change colors.

10

Another example of a free format assay is described by Chelsky, "Strategies for Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at the First Annual Conference of The Society for Biomolecular Screening in Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme
15   assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel would cause a color change throughout the gel. Thereafter, beads carrying combinatorial compounds via a photolinker were placed inside the gel and the compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

20

Yet another example is described by Salmon *et al.*, *Molecular Diversity 2,* 57-63 (1996). In this example, combinatorial libraries were screened for compounds that had cytotoxic effects on cancer cells growing in agar.

25   Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly,
30   such that the assays can be performed without the test samples running together.

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Binding Assays

For binding assays, the test compound is preferably a small molecule which binds to and occupies, for example, the active site of the phospholipase-like enzyme polypeptide, such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules.

In binding assays, either the test compound or the phospholipase-like enzyme polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horseradish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound which is bound to the phospholipase-like enzyme polypeptide can then be accomplished, for example, by direct counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a phospholipase-like enzyme poly-peptide can be determined without labeling either of the interactants. For example, a microphysiometer can be used to detect binding of a test compound with a phospholipase-like enzyme polypeptide. A microphysiometer (_e.g._, Cytosensor™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a phospholipase-like enzyme polypeptide (McConnell _et al._, _Science 257_, 1906-1912, 1992).

Determining the ability of a test compound to bind to a phospholipase-like enzyme polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, _Anal. Chem. 63_, 2338-2345, 1991, and Szabo _et al._, _Curr. Opin. Struct. Biol. 5_, 699-705, 1995). BIA is a technology for studying biospecific interactions in real time, without labeling any

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of the interactants (*e.g.*, BIAcore[TM]). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

5   In yet another aspect of the invention, a phospholipase-like enzyme polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent 5,283,317; Zervos *et al.*, *Cell 72*, 223-232, 1993; Madura *et al.*, *J. Biol. Chem. 268*, 12046-12054, 1993; Bartel *et al.*, *BioTechniques 14*, 920-924, 1993; Iwabuchi *et al.*, *Oncogene 8*, 1693-1696, 1993; and Brent W094/10300), to identify other

10   proteins which bind to or interact with the phospholipase-like enzyme polypeptide and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay

15   utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding a phospholipase-like enzyme polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of

20   the known transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ), which is operably linked to a transcriptional regulatory site responsive to the transcription factor.

25   Expression of the reporter gene can be detected, and cell colonies containing the functional transcription factor can be isolated and used to obtain the DNA sequence encoding the protein which interacts with the phospholipase-like enzyme polypeptide.

30   It may be desirable to immobilize either the phospholipase-like enzyme polypeptide (or polynucleotide) or the test compound to facilitate separation of bound from

unbound forms of one or both of the interactants, as well as to accommodate automation of the assay. Thus, either the phospholipase-like enzyme polypeptide (or polynucleotide) or the test compound can be bound to a solid support. Suitable solid supports include, but are not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach the enzyme polypeptide (or polynucleotide) or test compound to a solid support, including use of covalent and non-covalent linkages, passive absorption, or pairs of binding moieties attached respectively to the polypeptide (or polynucleotide) or test compound and the solid support. Test compounds are preferably bound to the solid support in an array, so that the location of individual test compounds can be tracked. Binding of a test compound to a phospholipase-like enzyme polypeptide (or polynucleotide) can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

In one embodiment, the phospholipase-like enzyme polypeptide is a fusion protein comprising a domain that allows the phospholipase-like enzyme polypeptide to be bound to a solid support. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed phospholipase-like enzyme polypeptide; the mixture is then incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a

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phospholipase-like enzyme polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated phospholipase-like enzyme polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N-hydroxysuccinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a phospholipase-like enzyme polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the phospholipase-like enzyme polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the phospholipase-like enzyme polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the phospholipase-like enzyme polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a phospholipase-like enzyme polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a phospholipase-like enzyme polypeptide or polynucleotide can be used in a cell-based assay system. A phospholipase-like enzyme polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a phospholipase-like enzyme polypeptide or polynucleotide is determined as described above.

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Enzyme Assays

Test compounds can be tested for the ability to increase or decrease the phospholipase or esterase activity of a human phospholipase-like enzyme polypeptide. phospholipase activity can be measured, for example, as described in Example 4.

Enzyme assays can be carried out after contacting either a purified phospholipase-like enzyme polypeptide, a cell membrane preparation, or an intact cell with a test compound. A test compound which decreases a phospholipase or esterase activity of a phospholipase-like enzyme polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for decreasing phospholipase-like enzyme activity. A test compound which increases a phospholipase or esterase activity of a human phospholipase-like enzyme polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential therapeutic agent for increasing human phospholipase-like enzyme activity.

Gene Expression

In another embodiment, test compounds which increase or decrease phospholipase-like enzyme gene expression are identified. A phospholipase-like enzyme polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the phospholipase-like enzyme polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide

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is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

The level of phospholipase-like enzyme mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide products of a phospholipase-like enzyme polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro* translation system by detecting incorporation of labeled amino acids into a phospholipase-like enzyme polypeptide.

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell which expresses a phospholipase-like enzyme polynucleotide can be used in a cell-based assay system. The phospholipase-like enzyme polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

Pharmaceutical Compositions

The invention also provides pharmaceutical compositions which can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a phospholipase-like enzyme polypeptide, phospholipase-like enzyme polynucleotide, ribozymes or antisense oligonucleotides, antibodies which specifically bind to a phospholipase-like enzyme polypeptide, or mimetics, activators, or inhibitors of a phospholipase-like enzyme polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline,

buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

5   In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Pharmaceutical compositions of the invention can be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal,
10   subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries,
15   suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to
20   obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired,
25   disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone,
30   carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to

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the tablets or dragee coatings for product identification or to characterize the quantity of active compound, *i.e.*, dosage.

5   Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid

10   polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous

15   injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or

20   liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are

25   generally known in the art.

The pharmaceutical compositions of the present invention can be manufactured in a manner that is known in the art, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or

30   lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric,

sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

Therapeutic Indications and Methods

Cancer. Human phospholipase-like enzyme can be regulated to treat cancer. Cancer is a disease fundamentally caused by oncogenic cellular transformation. There are several hallmarks of transformed cells that distinguish them from their normal counterparts and underlie the pathophysiology of cancer. These include uncontrolled cellular proliferation, unresponsiveness to normal death-inducing signals (immortalization), increased cellular motility and invasiveness, increased ability to recruit blood supply through induction of new blood vessel formation (angiogenesis), genetic instability, and dysregulated gene expression. Various combinations of these aberrant physiologies, along with the acquisition of drug-resistance frequently lead to an intractable disease state in which organ failure and patient death ultimately ensue.

Most standard cancer therapies target cellular proliferation and rely on the differential proliferative capacities between transformed and normal cells for their efficacy. This approach is hindered by the facts that several important normal cell types are also highly proliferative and that cancer cells frequently become resistant to

these agents. Thus, the therapeutic indices for traditional anti-cancer therapies rarely exceed 2.0.

The advent of genomics-driven molecular target identification has opened up the possibility of identifying new cancer-specific targets for therapeutic intervention that will provide safer, more effective treatments for cancer patients. Thus, newly discovered tumor-associated genes and their products can be tested for their role(s) in disease and used as tools to discover and develop innovative therapies. Genes playing important roles in any of the physiological processes outlined above can be characterized as cancer targets.

Genes or gene fragments identified through genomics can readily be expressed in one or more heterologous expression systems to produce functional recombinant proteins. These proteins are characterized *in vitro* for their biochemical properties and then used as tools in high-throughput molecular screening programs to identify chemical modulators of their biochemical activities. Activators and/or inhibitors of target protein activity can be identified in this manner and subsequently tested in cellular and *in vivo* disease models for anti-cancer activity. Optimization of lead compounds with iterative testing in biological models and detailed pharmacokinetic and toxicological analyses form the basis for drug development and subsequent testing in humans.

It is known that agents which increase phospholipase A2 activity stimulate the recruitment/activation of cells of the immune system. U.S. Patent No. 5,527,775. Based on this observation, the present invention provides methods for treating neoplastic diseases. Specifically, tumor necrosis, the killing of neoplastic cells and a reduction in the size of a neoplastic mass, can be induced in a mammal suffering from a neoplastic disease by administering an effective amount of an agent which stimulates human phospholipase-like enzyme activity or promotes arachidonic acid release, an indication of phospholipase-like enzyme activity. The stimulation of phospholipase-like enzyme activity results in the generation of eicosanoids, leading

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to the recruitment/activation of cell which promote an inflammatory response. The inflammatory response triggers the mammal's immune system to destroy the neoplastic cells. The response initiated by the treatment of the present invention results in a reduction in the severity of the neoplastic disease, a reduction of the tumor burden in the mammal, and a significant reduction in the number of neoplastic cells and neoplasm mass.

CNS Disorders. A_β-mediated neuronal cell degeneration is mediated by a bio-chemical cascade which requires activity of PLA2. U.S. Patent No. 5,707,821. Agents which selectively block PLA2 activity in neurons and/or glial cells and/or astrocytes or monocytes can be used to inhibit A_β-mediated neuronal degeneration, such as that which results from exposure of such cells to pathogenic forms of A_β as occurs in Alzheimer's Disease and related A_β-induced neurodegenerative conditions. It is likely, therefore, that inhibitors of human phospholipase-like enzyme also can be used to inhibit neuronal degeneration resulting from A_β toxicity.

CNS disorders which may be treated include brain injuries, cerebrovascular diseases and their consequences, Parkinson's disease, corticobasal degeneration, motor neuron disease, dementia, including ALS, multiple sclerosis, traumatic brain injury, stroke, post-stroke, post-traumatic brain injury, and small-vessel cerebrovascular disease. Dementias, such as Alzheimer's disease, vascular dementia, dementia with Lewy bodies, frontotemporal dementia and Parkinsonism linked to chromosome 17, frontotemporal dementias, including Pick's disease, progressive nuclear palsy, corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jakob dementia, HIV dementia, schizophrenia with dementia, and Korsakoff's psychosis also can be treated. Similarly, it may be possible to treat cognitive-related disorders, such as mild cognitive impairment, age-associated memory impairment, age-related cognitive decline, vascular cognitive impairment, attention deficit disorders, attention deficit hyperactivity disorders, and memory disturbances in children with learning disabilities, by regulating the activity of human phospholipase-like enzyme.

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Pain that is associated with CNS disorders also can be treated by regulating the activity of human phospholipase-like enzyme. Pain which can be treated includes that associated with central nervous system disorders, such as multiple sclerosis,

5   spinal cord injury, sciatica, failed back surgery syndrome, traumatic brain injury, epilepsy, Parkinson's disease, post-stroke, and vascular lesions in the brain and spinal cord (*e.g.*, infarct, hemorrhage, vascular malformation). Non-central neuro-pathic pain includes that associated with post mastectomy pain, reflex sympathetic dystrophy (RSD), trigeminal neuralgiaradioculopathy, post-surgical pain, HIV/AIDS

10 related pain, cancer pain, metabolic neuropathies (*e.g.*, diabetic neuropathy, vasculitic neuropathy secondary to connective tissue disease), paraneoplastic poly-neuropathy associated, for example, with carcinoma of lung, or leukemia, or lymphoma, or carcinoma of prostate, colon or stomach, trigeminal neuralgia, cranial neuralgias, and post-herpetic neuralgia. Pain associated with cancer and cancer

15 treatment also can be treated, as can headache pain (for example, migraine with aura, migraine without aura, and other migraine disorders), episodic and chronic tension-type headache, tension-type like headache, cluster headache, and chronic paroxysmal hemicrania.

20 *Allergy, anaphylaxis, asthma and inflammation.* It is now well-established that arachidonic acid (AA) is metabolized in mammals by two distinct pathways. U.S. Patent No. 5,070,207. The metabolism of arachidonic acid by cyclooxygenase enzymes results in the production of prostaglandins and thromboxanes. The physiological activity of the prostaglandins has already been amply elucidated in

25 recent years. It is now known that prostaglandins arise from the endoperoxides PGG_2 and PGH_2 by the cyclooxygenase pathway of arachidonic acid metabolism. These endoperoxides are also the precursors of the thromboxanes (Tx) A_2 and B_2. TxA_2 is a vasoconstrictor which stimulates platelet aggregation. In the normal situation, the vasoconstrictive and platelet aggregating properties of the throm-

30 boxanes are balanced by another product arising from the endoperoxides in the cyclooxygenase pathway, prostacyclin (PGI_2), which is a vasodilator with platelet

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aggregation inhibitory activity. In the event prostacyclin synthesis is impaired and/or platelet activation is enhanced, then thrombosis and vasoconstriction is favored. The role of prostanoids in haemostasis and thrombosis are reviewed by Gryglewski, *CRC Crit. Rev. Biochem.* 7, 291, 1980, and Smith, *Am. J. Pathol.* 99, 743, 1980. Cyclo-oxygenase metabolites are known to participate directly in the inflammatory response. See Higgs *et al.*, *Annals of Clinical Research 16*, 287-99, 1984. This is through their vasodepressor activities, participation in pain and fever, augmentation of peptide mediator vascular permeability and edema forming properties. Finally, various aspects of cell mediated immunity are influenced by cyclooxygenase products.

The other pathway of AA metabolism involves lipoxygenase enzymes and results in the production of a number of oxidative products called leukotrienes. The latter are designated by the LT nomenclature system, and the most significant products of the lipoxygenase metabolic pathway are the leukotrienes B_4, C_4, and D_4. The substance denominated slow-reacting substance of anaphylaxis (SRS-A) has been shown to consist of a mixture of leukotrienes, with LTC_4 and LTD_4 as the primary products and having varying amounts of other leukotriene metabolites . See Bach *et al.*, *J. Immun. 215*, 115-18, 1980; *Biochem. Biophys. Res. Commun.* 93, 1121-26, 1980.

The significance of these leukotrienes is that a great deal of evidence has been accumulated showing that leukotrienes participate in inflammatory reactions, exhibit chemotactic activities, stimulate lysosomal enzyme release and act as important factors in the immediate hypersensitivity reaction. It has been shown that LTC_4 and LTD_4 are potent bronchoconstrictors of the human bronchi (Dahlen *et al.*, *Nature 288*, 484-486 (1980) and Piper, *Int. Arch. Appl. Immunol.* 76, suppl. 1, 43 (1985)0, which stimulate the release of mucus from airways *in vitro* [Marom *et al.*, *Am. Rev. Resp. Dis. 126*, 449 (1982)], are potent vasodilators in skin [see Bisgaard *et al.*, *Prostaglandins 23*, 797 (1982)], and produce a wheal and flare response [Camp *et al.*, *Br. J. Pharmacol. 80*, 497 (1983)]. The nonpeptide leukotriene, LTB_4, is a powerful chemotactic factor for leukocytes [see A. W. Ford-Hutchinson, *J. Roy. Soc.*

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Med. 74, 831-833 (1981), which stimulates cell accumulation and affects vascular smooth muscle [see Bray, *Br. Med. Bull. 39,* 249 (1983)]. The activity of leukotrienes as mediators of inflammation and hypersensitivity is extensively reviewed in Bailey and Casey, *Ann. Reports Med. Chem., 19,* 87 (1986).

5

Phospholipase A2 is the critical rate limiting enzyme in the arachidonic acid (AA) cascade, because it is responsible for the hydrolysis of esterified AA from the C-2 position of membrane phospholipids. This reaction generates two products: (1) free AA which is then available for subsequent metabolism by either the

10   cyclooxygenase or lipoxygenase enzymes, and (2) lysophospholipid. When alkylarachidonoyl-glycerophosphatidylcholine is acted upon by the PLA2, the generation of platelet activating factor (PAF) is initiated; PAF is pro-inflammatory in its own right [see Wedmore *et al., Br. J. Pharmacol., 74,* 916-917 (1981)]. In this regard it may be noted that the anti-inflammatory steroids are thought to inhibit

15   eicosanoid synthesis by inducing the synthesis of a PLA2 inhibitory protein denominated macrocortin, lipomodulin or lipocortin [see Flower *et al., Nature, London, 278,* 456 (1979) and Hirata *et al., Proc. Natn. Acad. Sci. U.S.A., 77,* 2533 (1980)].

20   As the initial step leading to subsequent conversion of AA to the various eicosanoids by the cyclooxygenase and lipoxygenase pathways, the PLA2 -mediated release of AA from membrane phospholipids is a critical event in attempting to deal with the various physiological manifestations which are based on the activity of the eicosanoids and/or PAF. Thus, while PLA2 has been shown to be required for

25   platelet aggregation [Pickett *et al.,* Biochem. J., 160, 405 (1976)], cardiac contraction and excitation [Geisler *et al., Pharm. Res. Commun., 9,* 117 (1977)], as well as prostaglandin synthesis [Vogt, *Adv. Prostagl. Thromb. Res., 3,* 89 (1978)], the inhibition of PLA2 is indicated in the therapeutic treatment of both PAF induced or cyclooxygenase and/or lipoxygenase pathway product-mediated physiological

30   conditions. Thus, human phospholipase-like enzyme inhibitors are a rational

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approach to the prevention, removal or amelioration of such conditions as allergy, anaphylaxis, asthma and inflammation.

Allergy is a complex process in which environmental antigens induce clinically adverse reactions. The inducing antigens, called allergens, typically elicit a specific IgE response and, although in most cases the allergens themselves have little or no intrinsic toxicity, they induce pathology when the IgE response in turn elicits an IgE-dependent or T cell-dependent hypersensitivity reaction. Hypersensitivity reactions can be local or systemic and typically occur within minutes of allergen exposure in individuals who have previously been sensitized to an allergen. The hypersensitivity reaction of allergy develops when the allergen is recognized by IgE antibodies bound to specific receptors on the surface of effector cells, such as mast cells, basophils, or eosinophils, which causes the activation of the effector cells and the release of mediators that produce the acute signs and symptoms of the reactions. Allergic diseases include asthma, allergic rhinitis (hay fever), atopic dermatitis, and anaphylaxis.

Asthma is though to arise as a result of interactions between multiple genetic and environmental factors and is characterized by three major features: 1) intermittent and reversible airway obstruction caused by bronchoconstriction, increased mucus production, and thickening of the walls of the airways that leads to a narrowing of the airways, 2) airway hyperresponsiveness caused by a decreased control of airway caliber, and 3) airway inflammation. Certain cells are critical to the inflammatory reaction of asthma and they include T cells and antigen presenting cells, B cells that produce IgE, and mast cells, basophils, eosinophils, and other cells that bind IgE. These effector cells accumulate at the site of allergic reaction in the airways and release toxic products that contribute to the acute pathology and eventually to the tissue destruction related to the disorder. Other resident cells, such as smooth muscle cells, lung epithelial cells, mucus-producing cells, and nerve cells may also be abnormal in individuals with asthma and may contribute to the pathology. While the airway obstruction of asthma, presenting clinically as an intermittent wheeze and

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shortness of breath, is generally the most pressing symptom of the disease requiring immediate treatment, the inflammation and tissue destruction associated with the disease can lead to irreversible changes that eventually make asthma a chronic disabling disorder requiring long-term management.

5

Despite recent important advances in our understanding of the pathophysiology of asthma, the disease appears to be increasing in prevalence and severity (Gergen and Weiss, *Am. Rev. Respir. Dis. 146*, 823-24, 1992). It is estimated that 30-40% of the population suffer with atopic allergy, and 15% of children and 5% of adults in the

10   population suffer from asthma (Gergen and Weiss, 1992). Thus, an enormous burden is placed on our health care resources. However, both diagnosis and treatment of asthma are difficult. The severity of lung tissue inflammation is not easy to measure and the symptoms of the disease are often indistinguishable from those of respiratory infections, chronic respiratory inflammatory disorders, allergic rhinitis, or other

15   respiratory disorders. Often, the inciting allergen cannot be determined, making removal of the causative environmental agent difficult. Current pharmacological treatments suffer their own set of disadvantages. Commonly used therapeutic agents, such as beta activators, can act as symptom relievers to transiently improve pulmonary function, but do not affect the underlying inflammation. Agents that can

20   reduce the underlying inflammation, such as anti-inflammatory steroids, can have major drawbacks that range from immunosuppression to bone loss (Goodman and Gilman's THE PHARMACOLOGIC BASIS OF THERAPEUTICS, Seventh Edition, MacMillan Publishing Company, NY, USA, 1985). In addition, many of the present therapies, such as inhaled corticosteroids, are short-lasting, inconvenient to use, and

25   must be used often on a regular basis, in some cases for life, making failure of patients to comply with the treatment a major problem and thereby reducing their effectiveness as a treatment.

Because of the problems associated with conventional therapies, alternative treatment

30   strategies have been evaluated. Glycophorin A (Chu and Sharom, *Cell. Immunol. 145*, 223-39, 1992), cyclosporin (Alexander *et al.*, *Lancet 339*, 324-28, 1992), and a

nonapeptide fragment of IL-2 (Zav'yalov *et al.*, *Immunol. Lett. 31*, 285-88, 1992) all inhibit interleukin-2 dependent T lymphocyte proliferation; however, they are known to have many other effects. For example, cyclosporin is used as a immuno-suppressant after organ transplantation. While these agents may represent alterna-tives to steroids in the treatment of asthmatics, they inhibit interleukin-2 dependent T lymphocyte proliferation and potentially critical immune functions associated with homeostasis. Other treatments that block the release or activity of mediators of bronchochonstriction, such as cromones or anti-leukotrienes, have recently been introduced for the treatment of mild asthma, but they are expensive and not effective in all patients and it is unclear whether they have any effect on the chronic changes associated with asthmatic inflammation. What is needed in the art is the identifi-cation of a treatment that can act in pathways critical to the development of asthma that both blocks the episodic attacks of the disorder and preferentially dampens the hyperactive allergic immune response without immunocompromising the patient.

Cardiovascular Disease. Evidence has accumulated for a potential role of PLA2 in myocardial injury to the ischemic heart. U.S. Patent No. 5,208,244. For example, it has been shown that endogenous fatty acids increase several fold in an ischemic dog heart *in vivo* and in isolated perfused ischemic rat and dog hearts. See, for example, van der Vusse et al, Circ. Res. 50, 538-46 (1982); Chien et al, Circ. Res., 54, 313-22 (1984); Burton et al, Am. J. Pathol., 124, 238-45 (1985). It has also been shown that lysophospholipids accumulate 2 to 3 fold in the ischemic cat heart *in vivo*, Corr. et al, J. Clin. Invest., 83, 927-36 (1989). The accumulation of the products of phos-pholipid hydrolysis implies that a PLA2 may become activated in the ischemic heart.

Lysophospholipids have also been implicated as potential mediators of sudden cardiac death, Corr et al, "Lethal Arrhythmias Resulting from Myocardial Ischemia and Infarction", Rosen & Patti, eds., Kluwer Academic Publishers, Boston, 91-014 (1989). The addition of lysophospholipids to normoxic myocardial tissue *in vitro* induces electrophysiological alterations that are similar to those observed in the ischemic heart *in vivo*. Corr et al, Circ. Res, 55, 135-54 (1984).

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Most importantly, lysophospholipid accumulation in the ischemic dog heart in vivo has been correlated with the frequency of cardiac arrhythmias, Kinnaird et al, Lipids, 23, 32-35 (1988). Furthermore, it is known that the carnitine acyltransferase 1 inhibitor, 2-[5-(4-chlorophenyl)-pentyl]-oxirane-2-carboxylate (POCA), prevents the onset of ventricular fibrillation and ventricular tachycardia and inhibits the accumulation of lysophospholipids (and long-chain aoyloarnitines) in the ischemic cat heart *in vivo*. Corr et al, J. Clin. Invest., 83, 927-36 (1989).

Accelerated phospholipid catabolism by PLA2 has also been implicated as a cause of infarct damage in the ischemic heart. In the ischemic heart, ATP levels decrease. Treatment of rat neonatal myocytes with the glycolytic inhibitor iodoacetate lowers the levels of ATP which results in the release of arachidonic acid and morphological alterations of the myocytes, Chien et al, J. Clin. Invest., 75, 1770-80 (1985). One PLA2 inhibitor (U26,384) prevented the release of arachidonic acid, phospholipid degradation, sarcolemmal membrane defects and the release of creatine kinase that was induced by the treatment of rat neonatal myocytes with iodoacetate, Sen et al, J. Clin. Invest., 82, 1333-38 (1988).

Therefore, inhibition of human phospholipase-like enzyme activity, without inhibiting other mammalian PLA2 enzymes, to prevent the accumulation of arrhythmogenic lysophospholipids in an ischemic heart is a potential therapeutic approach for the prevention of arrhythmias, infarct damage and sudden death.

Chronic obstructive pulmonary disease. Inhibitors of human phospholipase-like enzyme also can be used to treat other conditions mediated by products of the oxidation of arachidonic acid, such as chronic obstructive pulmonary disease, emphysema, and acute respiratory distress syndrome. U.S. Patent No. 5,508,302. Chronic obstructive pulmonary (or airways) disease (COPD) is a condition defined physiologically as airflow obstruction that generally results from a mixture of emphysema and peripheral airway obstruction due to chronic bronchitis (Senior &

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Shapiro, *Pulmonary Diseases and Disorders*, 3d ed., New York, McGraw-Hill, 1998, pp. 659-681, 1998; Barnes, *Chest 117*, 10S-14S, 2000). Emphysema is characterized by destruction of alveolar walls leading to abnormal enlargement of the air spaces of the lung. Chronic bronchitis is defined clinically as the presence of chronic productive cough for three months in each of two successive years. In COPD, airflow obstruction is usually progressive and is only partially reversible. By far the most important risk factor for development of COPD is cigarette smoking, although the disease does occur in non-smokers.

Chronic inflammation of the airways is a key pathological feature of COPD (Senior & Shapiro, 1998). The inflammatory cell population comprises increased numbers of macrophages, neutrophils, and $CD8^+$ lymphocytes. Inhaled irritants, such as cigarette smoke, activate macrophages which are resident in the respiratory tract, as well as epithelial cells leading to release of chemokines (*e.g.*, interleukin-8) and other chemotactic factors. These chemotactic factors act to increase the neutrophil/-monocyte trafficking from the blood into the lung tissue and airways. Neutrophils and monocytes recruited into the airways can release a variety of potentially damaging mediators such as proteolytic enzymes and reactive oxygen species. Matrix degradation and emphysema, along with airway wall thickening, surfactant dysfunction, and mucus hypersecretion, all are potential sequelae of this inflammatory response that lead to impaired airflow and gas exchange.

Obesity. Obesity, too, may be treated by regulating human phospholipase-like enzyme. Obesity and overweight are defined as an excess of body fat relative to lean body mass. An increase in caloric intake or a decrease in energy expenditure or both can bring about this imbalance leading to surplus energy being stored as fat. Obesity is associated with important medical morbidities and an increase in mortality. The causes of obesity are poorly understood and may be due to genetic factors, environmental factors or a combination of the two to cause a positive energy balance. In contrast, anorexia and cachexia are characterized by an imbalance in energy intake versus energy expenditure leading to a negative energy balance and weight loss.

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Agents that either increase energy expenditure and/or decrease energy intake, absorption or storage would be useful for treating obesity, overweight, and associated comorbidities. Agents that either increase energy intake and/or decrease energy expenditure or increase the amount of lean tissue would be useful for treating
5   cachexia, anorexia and wasting disorders.

This gene, translated proteins and agents which modulate this gene or portions of the gene or its products are useful for treating obesity, overweight, anorexia, cachexia, wasting disorders, appetite suppression, appetite enhancement, increases or decreases
10   in satiety, modulation of body weight, and/or other eating disorders such as bulimia. Also this gene, translated proteins and agents which modulate this gene or portions of the gene or its products are useful for treating obesity/overweight-associated comor-bidities including hypertension, type 2 diabetes, coronary artery disease, hyper-lipidemia, stroke, gallbladder disease, gout, osteoarthritis, sleep apnea and respiratory
15   problems, some types of cancer including endometrial, breast, prostate, and colon cancer, thrombolic disease, polycystic ovarian syndrome, reduced fertility, compli-cations of pregnancy, menstrual irregularities, hirsutism, stress incontinence, and depression.

20   _Diabetes_. Diabetes mellitus is a common metabolic disorder characterized by an abnormal elevation in blood glucose, alterations in lipids and abnormalities (complications) in the cardiovascular system, eye, kidney and nervous system. Diabetes is divided into two separate diseases: type 1 diabetes (juvenile onset), which results from a loss of cells which make and secrete insulin, and type 2 diabetes
25   (adult onset), which is caused by a defect in insulin secretion and a defect in insulin action.

Type 1 diabetes is initiated by an autoimuune reaction that attacks the insulin secreting cells (beta cells) in the pancreatic islets. Agents that prevent this reaction
30   from occurring or that stop the reaction before destruction of the beta cells has been accomplished are potential therapies for this disease. Other agents that induce beta

cell proliferation and regeneration also are potential therapies.

Type II diabetes is the most common of the two diabetic conditions (6% of the population). The defect in insulin secretion is an important cause of the diabetic

5   condition and results from an inability of the beta cell to properly detect and respond to rises in blood glucose levels with insulin release. Therapies that increase the response by the beta cell to glucose would offer an important new treatment for this disease.

10   The defect in insulin action in Type II diabetic subjects is another target for therapeutic intervention. Agents that increase the activity of the insulin receptor in muscle, liver, and fat will cause a decrease in blood glucose and a normalization of plasma lipids. The receptor activity can be increased by agents that directly stimulate the receptor or that increase the intracellular signals from the receptor. Other

15   therapies can directly activate the cellular end process, *i.e.* glucose transport or various enzyme systems, to generate an insulin-like effect and therefore a produce beneficial outcome. Because overweight subjects have a greater susceptibility to Type II diabetes, any agent that reduces body weight is a possible therapy.

20   Both Type I and Type diabetes can be treated with agents that mimic insulin action or that treat diabetic complications by reducing blood glucose levels. Likewise, agents that reduces new blood vessel growth can be used to treat the eye complications that develop in both diseases.

25   This invention further pertains to the use of novel agents identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a phospholipase-

30   like enzyme polypeptide binding molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent.

Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

5

A reagent which affects phospholipase-like enzyme activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce phospholipase-like enzyme activity. The reagent preferably binds to an expression product of a human phospholipase-like enzyme gene. If the expression product is a protein, the reagent is preferably an antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells which have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

15   In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about $0.5\,\mu g$ of DNA per 16 nmole of liposome delivered to about 10^6 cells, more preferably about $1.0\,\mu g$ of DNA per 16 nmole of liposome delivered to about 10^6 cells, and even more preferably about $2.0\,\mu g$ of DNA per 16 nmol of liposome delivered to about 10^6 cells. Preferably, a liposome is between about 100 and

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500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods which are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1 µg to about 10 µg of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5 µg to about 5 µg of polynucleotides are combined with about 8 nmol liposomes, and even more preferably about 1.0 µg of polynucleotides is combined with about 8 nmol liposomes.

In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis *et al. Trends in Biotechnol. 11*, 202-05 (1993); Chiou *et al.*, GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem. 263*, 621-24 (1988); Wu *et al.*, *J. Biol. Chem. 269*, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 87*, 3655-59 (1990); Wu *et al.*, *J. Biol. Chem. 266*, 338-42 (1991).

Determination of a Therapeutically Effective Dose

The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of

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active ingredient which increases or decreases phospholipase-like enzyme activity relative to the phospholipase-like enzyme activity which occurs in the absence of the therapeutically effective dose.

5   For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

10

Therapeutic efficacy and toxicity, e.g., ED_{50} (the dose therapeutically effective in 50% of the population) and LD_{50} (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it 15   can be expressed as the ratio, LD_{50}/ED_{50}.

Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is 20   preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to 25   the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and 30   tolerance/response to therapy. Long-acting pharmaceutical compositions can be

administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of poly-nucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

Effective *in vivo* dosages of an antibody are in the range of about 5 µg to about 50 µg/kg, about 50 µg to about 5 mg/kg, about 100 µg to about 500 µg/kg of patient body weight, and about 200 to about 250 µg/kg of patient body weight. For administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of DNA.

If the expression product is mRNA, the reagent is preferably an antisense oligo-nucleotide or a ribozyme. Polynucleotides which express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

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Preferably, a reagent reduces expression of a phospholipase-like enzyme gene or the activity of a phospholipase-like enzyme polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the
5   reagent.   The effectiveness of the mechanism chosen to decrease the level of expression of a phospholipase-like enzyme gene or the activity of a phospholipase-like enzyme polypeptide can be assessed using methods well known in the art, such as hybridization of nucleotide probes to phospholipase-like enzyme-specific mRNA, quantitative RT-PCR, immunologic detection of a phospholipase-like enzyme
10   polypeptide, or measurement of phospholipase-like enzyme activity.

In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate thera-peutic agents. Selection of the appropriate agents for use in combination therapy can
15   be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above.   Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.
20

Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

25   _Diagnostic Methods_

Human phospholipase-like enzyme also can be used in diagnostic assays for detecting diseases and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences which encode the
30   enzyme. For example, differences can be determined between the cDNA or genomic sequence encoding phospholipase-like enzyme in individuals afflicted with a disease

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and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

5   Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments can be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer can be used with a double-stranded PCR product or a single-stranded template

10   molecule generated by a modified PCR. The sequence determination is performed by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

Genetic testing based on DNA sequence differences can be carried out by detection

15   of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions

20   according to their specific melting or partial melting temperatures (*see, e.g.*, Myers *et al.*, *Science 230*, 1242, 1985). Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (*e.g.*, Cotton *et al.*, *Proc. Natl. Acad. Sci. USA 85*, 4397-4401, 1985). Thus, the detection of a specific DNA sequence can be performed

25   by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes and Southern blotting of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

30   Altered levels of a phospholipase-like enzyme also can be detected in various tissues. Assays used to detect levels of the receptor polypeptides in a body sample, such as

blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

5   All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

10

EXAMPLE 1

Detection of phospholipase-like enzyme activity

15   The polynucleotide of SEQ ID NO: 1 is inserted into the expression vector pCEV4 and the expression vector pCEV4-phospholipase-like enzyme polypeptide obtained is transfected into human embryonic kidney 293 cells. From these cells extracts are obtained. Human phospholipase-like enzyme activity is determined by an *in vitro* hydrolysis assay with vesicles containing 1-palmitoyl-2-[1-14C]oleoyl phos-

20   phatidylcholine (Sigma Chemical Co.). Phospholipase activity is demonstrates by analysis of the cleavage products isolated from the hydrolysis reaction mixture.

Vesicles containing 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine (Amersham Corp.) are prepared by mixing 2.0 µCi of the radiolabeled phospholipid with 12.5 mg

25   of unlabeled 1-palmitoyl-2-oleoyl phosphatidylcholine and drying the mixture under N_2. Two and a half ml of 150 mM Tris-HCl, pH 7.5, is added, and the mixture is sonicated and centrifuged. The supernatant may be stored at 4 °C. The final reaction mixture contains 0.25 ml of Hanks buffered salt solution supplemented with 2.0 mM taurochenodeoxycholate, 1.0% bovine serum albumin, 1.0 mM $CaCl_2$, pH 7.4,

30   150 µg of 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine vesicles, and various amount of human phospholipase-like enzyme diluted in PBS. After incubation for

30 min at 37°C, 20 µg each of lyso-phosphatidylcholine and oleic acid are added as carriers and each sample is extracted for total lipids.

5   The lipids are separated by thin layer chromatography, using a two solvent system of chloroform:methanol:acetic acid:water (65:35:8:4) until the solvent front is halfway up the plate, the process is then continued with hexane:ether:acetic acid (86:16:1) until the solvent front is at the top of the plate. The lipid-containing areas are visualized with I2 vapor; the spots are scraped, and their radioactivity is determined by scintillation counting. The amount of radioactivity released as fatty acids will

10 increase. The cell extracts mentioned above are added to the assay mixture while the amount of radioactivity released as lyso-phosphatidylcholine will remain low. This demonstrates that human phospholipase-like enzyme cleaves at the sn-2 and not the sn-1 position, exhibiting phospholipase A2 activity. It is shown that the polypeptide of SEQ ID NO: 2 has a phospholipase-like enzyme activity.

15

EXAMPLE 2

Expression of recombinant human phospholipase-like enzyme

20 The *Pichia pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) is used to produce large quantities of recombinant human phospholipase-like polypeptides in yeast. The phospholipase-like enzyme-encoding DNA sequence is derived from SEQ ID NO: 1. Before insertion into vector pPICZB, the DNA sequence is modified by well known methods in such a way that it contains at its 5'-end an initiation codon

25 and at its 3'-end an enterokinase cleavage site, a His6 reporter tag and a termination codon. Moreover, at both termini recognition sequences for restriction endonucleases are added and after digestion of the multiple cloning site of pPICZ B with the corresponding restriction enzymes the modified DNA sequence is ligated into pPICZB. This expression vector is designed for inducible expression in *Pichia*

30 *pastoris*, driven by a yeast promoter. The resulting pPICZ/md-His6 vector is used to transform the yeast.

The yeast is cultivated under usual conditions in 5 liter shake flasks and the recombinantly produced protein isolated from the culture by affinity chromatography (Ni-NTA-Resin) in the presence of 8 M urea. The bound polypeptide is eluted with buffer, pH 3.5, and neutralized. Separation of the polypeptide from the His6 reporter tag is accomplished by site-specific proteolysis using enterokinase (Invitrogen, San Diego, CA) according to manufacturer's instructions. Purified human phospholipase-like enzyme polypeptide is obtained.

EXAMPLE 3

Identification of test compounds that bind to phospholipase-like enzyme polypeptides

Purified phospholipase-like enzyme polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. Human phospholipase-like enzyme polypeptides comprise the amino acid sequence shown in SEQ ID NO: 2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a phospholipase-like enzyme polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound which increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to a phospholipase-like enzyme polypeptide.

EXAMPLE 4

Identification of a test compound which decreases phospholipase-like enzyme gene expression

A test compound is administered to a culture of human cells transfected with a phospholipase-like enzyme expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells which have not been transfected is incubated for the same time without the test compound to provide a negative control.

RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem. 18*, 5294-99, 1979). Northern blots are prepared using 20 to 30 µg total RNA and hybridized with a ^{32}P-labeled phospholipase-like enzyme-specific probe at 65°C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides selected from the complement of SEQ ID NO: 2. A test compound which decreases the phospholipase-like enzyme-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of phospholipase-like enzyme gene expression.

EXAMPLE 5

Identification of a test compound which decreases phospholipase-like enzyme activity

A test compound is administered to a culture of human cells transfected with a phospholipase-like enzyme expression construct and incubated at 37°C for 10 to 45 minutes. A culture of the same type of cells which have not been transfected is incubated for the same time without the test compound to provide a negative control.

A test compound which decreases the phospholipase activity of the phospholipase-like enzyme relative to the phospholipase activity in the absence of the test compound is identified as an inhibitor of phospholipase-like enzyme activity.

EXAMPLE 6

Identification of molecules which interact with human phospholipase-like enzyme

Human phospholipase-like enzyme or biologically active fragments thereof are labeled with ^{125}1 Bolton-Hunter reagent (Bolton *et al.*, *Biochem. J. 133*, 529, 1973). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled human phospholipase-like enzyme, washed, and any wells with labeled human phospholipase-like enzyme complex are assayed. Data obtained using different concentrations of human phospholipase-like enzyme are used to calculate values for the number, affinity, and association of human phospholipase-like enzyme with the candidate molecules.

EXAMPLE 7

Tissue-specific expression of human phospholipase-like enzyme

The qualitative expression pattern of human phospholipase-like enzyme in various tissues is determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). To demonstrate that human phospholipase-like enzyme is involved in cancer, expression is determined in the following tissues: adrenal gland, bone marrow, brain, cerebellum, colon, fetal brain, fetal liver, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, trachea, uterus, and peripheral blood lymphocytes. Expression in the following cancer cell lines also is determined: DU-145 (prostate), NCI-H125 (lung), HT-29 (colon), COLO-205 (colon), A-549 (lung), NCI-H460 (lung), HT-116 (colon), DLD-1 (colon), MDA-MD-231 (breast), LS174T (colon), ZF-75 (breast), MDA-MN-435 (breast), HT-1080, MCF-7 (breast), and U87. Matched pairs of malignant and normal tissue from the same patient also are tested.

To demonstrate that human phospholipase-like enzyme is involved in CNS disorders, the following tissues are screened: fetal and adult brain, muscle, heart, lung, kidney, liver, thymus, testis, colon, placenta, trachea, pancreas, kidney, gastric mucosa, colon, liver, cerebellum, skin, cortex (Alzheimer's and normal), hypothalamus,

5   cortex, amygdala, cerebellum, hippocampus, choroid, plexus, thalamus, and spinal cord.

To demonstrate that human phospholipase-like enzyme is involved in the disease process of COPD, the initial expression panel consists of RNA samples from

10   respiratory tissues and inflammatory cells relevant to COPD: lung (adult and fetal), trachea, freshly isolated alveolar type II cells, cultured human bronchial epithelial cells, cultured small airway epithelial cells, cultured bronchial sooth muscle cells, cultured H441 cells (Clara-like), freshly isolated neutrophils and monocytes, and cultured monocytes (macrophage-like). Body map profiling also is carried out, using

15   total RNA panels purchased from Clontech. The tissues are adrenal gland, bone marrow, brain, colon, heart, kidney, liver, lung, mammary gland, pancreas, prostate, salivary gland, skeletal muscle, small intestine, spleen, stomach, testis, thymus, trachea, thyroid, and uterus. As a final step, the expression of human phospholipase-like enzyme in cells derived from normal individuals with the expression of cells

20   derived from COPD-affected individuals is compared.

To demonstrate that human phospholipase-like enzyme is involved in the disease process of diabetes, the following whole body panel is screened to show predominant or relatively high expression: subcutaneous and mesenteric adipose tissue, adrenal

25   gland, bone marrow, brain, colon, fetal brain, heart, hypothalamus, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid, trachea, and uterus. Human islet cells and an islet cell library also are tested. As a final step, the expression of human phospholipase-like enzyme in cells derived from normal individuals with the

30   expression of cells derived from diabetic individuals is compared.

To demonstrate that human phospholipase-like enzyme is involved in the disease process of obesity, expression is determined in the following tissues: subcutaneous adipose tissue, mesenteric adipose tissue, adrenal gland, bone marrow, brain (cerebellum, spinal cord, cerebral cortex, caudate, medulla, substantia nigra, and putamen), colon, fetal brain, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle small intestine, spleen, stomach, testes, thymus, thyroid trachea, and uterus. Neuroblastoma cell lines SK-Nr-Be (2), Hr, Sk-N-As, HTB-10, IMR-32, SNSY-5Y, T3, SK-N-D2, D283, DAOY, CHP-2, U87MG, BE(2)C, T986, KANTS, MO59K, CHP234, C6 (rat), SK-N-F1, SK-PU-DW, PFSK-1, BE(2)M17, and MCIXC also are tested for human phospholipase-like enzyme expression. As a final step, the expression of human phospholipase-like enzyme in cells derived from normal individuals with the expression of cells derived from obese individuals is compared.

Quantitative expression profiling. Quantitative expression profiling is performed by the form of quantitative PCR analysis called "kinetic analysis" firstly described in Higuchi *et al.*, *BioTechnology 10*, 413-17, 1992, and Higuchi *et al.*, *BioTechnology 11*, 1026-30, 1993. The principle is that at any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies.

If the amplification is performed in the presence of an internally quenched fluorescent oligonucleotide (TaqMan probe) complementary to the target sequence, the probe is cleaved by the 5'-3' endonuclease activity of Taq DNA polymerase and a fluorescent dye released in the medium (Holland *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 88*, 7276-80, 1991). Because the fluorescence emission will increase in direct proportion to the amount of the specific amplified product, the exponential growth phase of PCR product can be detected and used to determine the initial template concentration (Heid *et al.*, *Genome Res. 6*, 986-94, 1996, and Gibson *et al.*, *Genome Res. 6*, 995-1001, 1996).

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The amplification of an endogenous control can be performed to standardize the amount of sample RNA added to a reaction. In this kind of experiment, the control of choice is the 18S ribosomal RNA. Because reporter dyes with differing emission spectra are available, the target and the endogenous control can be independently

5   quantified in the same tube if probes labeled with different dyes are used.

All "real time PCR" measurements of fluorescence are made in the ABI Prism 7700.

RNA extraction and cDNA preparation. Total RNA from the tissues listed above are

10   used for expression quantification. RNAs labeled "from autopsy" are extracted from autoptic tissues with the TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol.

Fifty μg of each RNA are treated with DNase I for 1 hour at 37°C in the following

15   reaction mix: 0.2 U/μl RNase-free DNase I (Roche Diagnostics, Germany); 0.4 U/μl RNase inhibitor (PE Applied Biosystems, CA); 10 mM Tris-HCl pH 7.9; 10mM $MgCl_2$; 50 mM NaCl; and 1 mM DTT.

After incubation, RNA is extracted once with 1 volume of phenol:chloroform:-

20   isoamyl alcohol (24:24:1) and once with chloroform, and precipitated with 1/10 volume of 3 M NaAcetate, pH5.2, and 2 volumes of ethanol.

Fifty μg of each RNA from the autoptic tissues are DNase treated with the DNA-free kit purchased from Ambion (Ambion, TX). After resuspension and spectro-

25   photometric quantification, each sample is reverse transcribed with the TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA) according to the manufacturer's protocol. The final concentration of RNA in the reaction mix is 200-ng/μL. Reverse transcription is carried out with 2.5μM of random hexamer primers.

30

TaqMan quantitative analysis. Specific primers and probe are designed according to

the recommendations of PE Applied Biosystems, using the sequences disclosed herein. Probes can be labeled at the 5' end with FAM (6-carboxyfluorescein) and at the 3' end with TAMRA (6-carboxytetramethylrhodamine). Quantification experiments are performed on 10 ng of reverse transcribed RNA from each sample. Each

5   determination is done in triplicate.

Total cDNA content is normalized with the simultaneous quantification (multiplex PCR) of the 18S ribosomal RNA using the Pre-Developed TaqMan Assay Reagents (PDAR) Control Kit (PE Applied Biosystems, CA).

10

The assay reaction mix is as follows: 1X final TaqMan Universal PCR Master Mix (from 2X stock) (PE Applied Biosystems, CA); 1X PDAR control – 18S RNA (from 20X stock); 300 nM forward primer; 900 nM reverse primer; 200 nM probe; 10 ng cDNA; and water to 25 µl.

15

Each of the following steps are carried out once: pre PCR, 2 minutes at 50°C, and 10 minutes at 95°C. The following steps are carried out 40 times: denaturation, 15 seconds at 95°C, annealing/extension, 1 minute at 60°C.

20   The experiment is performed on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, CA). At the end of the run, fluorescence data acquired during PCR are processed as described in the ABI Prism 7700 user's manual in order to achieve better background subtraction as well as signal linearity with the starting target quantity.

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EXAMPLE 8

Diabetes: In vivo testing of compounds/target validation

5   1.   Glucose Production

Over-production of glucose by the liver, due to an enhanced rate of gluconeo-genesis, is the major cause of fasting hyperglycemia in diabetes. Overnight fasted normal rats or mice have elevated rates of gluconeogenesis as do streptozotocin-induced diabetic rats or mice fed ad libitum. Rats are made diabetic with a single intravenous injection of 40 mg/kg of streptozotocin while C57BL/KsJ mice are given 40-60 mg/kg i.p. for 5 consecutive days. Blood glucose is measured from tail-tip blood and then compounds are administered via different routes (p.o., i.p., i.v., s.c.). Blood is collected at various times thereafter and glucose measured. Alternatively, compounds are administered for several days, then the animals are fasted overnight, blood is collected and plasma glucose measured. Compounds that inhibit glucose production will decrease plasma glucose levels compared to the vehicle-treated control group.

2.   Insulin Sensitivity

Both ob/ob and db/db mice as well as diabetic Zucker rats are hyperglycemic, hyperinsulinemic and insulin resistant. The animals are pre-bled, their glucose levels measured, and then they are grouped so that the mean glucose level is the same for each group. Compounds are administered daily either q.d. or b.i.d. by different routes (p.o., i.p., s.c.) for 7-28 days. Blood is collected at various times and plasma glucose and insulin levels determined. Compounds that improve insulin sensitivity in these models will decrease both plasma glucose and insulin levels when compared to the vehicle-treated control group.

3.   Insulin Secretion

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, compounds are administered by different routes (p.o., i.p., s.c. or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1 g/kg), bled again after 15, 30, 60 and 90 minutes and plasma glucose levels determined. Compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, test compounds which regulate pristanoyl-CoA oxidase-like enzyme are administered by different routes (p.o., i.p., s.c., or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4 g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Test compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1g/kg), bled again after 15, 30, 60, and 90 minutes and plasma glucose levels determined. Test compounds that increase insulin levels will

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decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

4.   Glucose Production

Over-production of glucose by the liver, due to an enhanced rate of gluconeogenesis, is the major cause of fasting hyperglycemia in diabetes. Overnight fasted normal rats or mice have elevated rates of gluconeogenesis as do streptozotocin-induced diabetic rats or mice fed ad libitum. Rats are made diabetic with a single intravenous injection of 40 mg/kg of streptozotocin while C57BL/KsJ mice are given 40-60 mg/kg i.p. for 5 consecutive days. Blood glucose is measured from tail-tip blood and then compounds are administered via different routes (p.o., i.p., i.v., s.c.). Blood is collected at various times thereafter and glucose measured. Alternatively, compounds are administered for several days, then the animals are fasted overnight, blood is collected and plasma glucose measured. Compounds that inhibit glucose production will decrease plasma glucose levels compared to the vehicle-treated control group.

5.   Insulin Sensitivity

Both ob/ob and db/db mice as well as diabetic Zucker rats are hyperglycemic, hyperinsulinemic and insulin resistant. The animals are pre-bled, their glucose levels measured, and then they are grouped so that the mean glucose level is the same for each group. Compounds are administered daily either q.d. or b.i.d. by different routes (p.o., i.p., s.c.) for 7-28 days. Blood is collected at various times and plasma glucose and insulin levels determined. Compounds that improve insulin sensitivity in these models will decrease both plasma glucose and insulin levels when compared to the vehicle-treated control group.

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6.   Insulin Secretion

Compounds that enhance insulin secretion from the pancreas will increase plasma insulin levels and improve the disappearance of plasma glucose following the administration of a glucose load. When measuring insulin levels, compounds are administered by different routes (p.o., i.p., s.c. or i.v.) to overnight fasted normal rats or mice. At the appropriate time an intravenous glucose load (0.4g/kg) is given, blood is collected one minute later. Plasma insulin levels are determined. Compounds that enhance insulin secretion will increase plasma insulin levels compared to animals given only glucose. When measuring glucose disappearance, animals are bled at the appropriate time after compound administration, then given either an oral or intraperitoneal glucose load (1g/kg), bled again after 15, 30, 60 and 90 minutes and plasma glucose levels determined. Compounds that increase insulin levels will decrease glucose levels and the area-under-the glucose curve when compared to the vehicle-treated group given only glucose.

EXAMPLE 9

Proliferation inhibition assay: Antisense oligonucleotides suppress the growth of cancer cell lines

The cell line used for testing is the human colon cancer cell line HCT116. Cells are cultured in RPMI-1640 with 10-15% fetal calf serum at a concentration of 10,000 cells per milliliter in a volume of 0.5 ml and kept at 37°C in a 95% air/5%CO$_2$ atmosphere.

Phosphorothioate oligoribonucleotides are synthesized on an Applied Biosystems Model 380B DNA synthesizer using phosphoroamidite chemistry. A sequence of 24 bases complementary to the nucleotides at position 1 to 24 of SEQ ID NO: 1 is used as the test oligonucleotide. As a control, another (random) sequence is used: 5'-TCA

ACT GAC TAG ATG TAC ATG GAC-3'. Following assembly and deprotection, oligonucleotides are ethanol-precipitated twice, dried, and suspended in phosphate buffered saline at the desired concentration. Purity of the oligonucleotides is tested by capillary gel electrophoresis and ion exchange HPLC. The purified oligo-nucleotides are added to the culture medium at a concentration of 10 μM once per day for seven days.

The addition of the test oligonucleotide for seven days results in significantly reduced expression of human phospholipase-like enzyme as determined by Western blotting. This effect is not observed with the control oligonucleotide. After 3 to 7 days, the number of cells in the cultures is counted using an automatic cell counter. The number of cells in cultures treated with the test oligonucleotide (expressed as 100%) is compared with the number of cells in cultures treated with the control oligonucleotide. The number of cells in cultures treated with the test oligonucleotide is not more than 30% of control, indicating that the inhibition of human phospholipase-like enzyme has an anti-proliferative effect on cancer cells.

EXAMPLE 10

In vivo testing of compounds/target validation

1.   **Acute Mechanistic Assays**

1.1.   Reduction in Mitogenic Plasma Hormone Levels

This non-tumor assay measures the ability of a compound to reduce either the endogenous level of a circulating hormone or the level of hormone produced in response to a biologic stimulus. Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.). At a pre-determined time after administration of test compound, blood plasma is collected. Plasma is assayed for levels of the hormone of interest.

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If the normal circulating levels of the hormone are too low and/or variable to provide consistent results, the level of the hormone may be elevated by a pre-treatment with a biologic stimulus (i.e., LHRH may be injected i.m. into mice at a dosage of 30 ng/mouse to induce a burst of testosterone synthesis). The timing of plasma collection would be adjusted to coincide with the peak of the induced hormone response. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value $\leq$ 0.05 compared to the vehicle control group.

1.2. *Hollow Fiber Mechanism of Action Assay*

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol, these may include assays for gene expression (bDNA, PCR, or Taqman), or a specific biochemical activity (i.e., cAMP levels. Results are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at p $\leq$ 0.05 as compared to the vehicle control group.

2.   Subacute Functional *In Vivo* Assays

2.1. *Reduction in Mass of Hormone Dependent Tissues*

This is another non-tumor assay that measures the ability of a compound to reduce the mass of a hormone dependent tissue (i.e., seminal vesicles in males and uteri in females). Rodents are administered test compound (p.o., i.p., i.v., i.m., or s.c.) according to a

predetermined schedule and for a predetermined duration (i.e., 1 week). At termination of the study, animals are weighed, the target organ is excised, any fluid is expressed, and the weight of the organ is recorded. Blood plasma may also be collected. Plasma may be assayed for levels of a hormone of interest or for levels of test agent. Organ weights may be directly compared or they may be normalized for the body weight of the animal. Compound effects are compared to a vehicle-treated control group. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test. Significance is p value ≤ 0.05 compared to the vehicle control group.

2.2. *Hollow Fiber Proliferation Assay*

Hollow fibers are prepared with desired cell line(s) and implanted intraperitoneally and/or subcutaneously in rodents. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Fibers are harvested in accordance with specific readout assay protocol. Cell proliferation is determined by measuring a marker of cell number (i.e., MTT or LDH). The cell number and change in cell number from the starting inoculum are analyzed by Student's t-test or Rank Sum test after the variance between groups is compared by an F-test, with significance at $p \leq 0.05$ as compared to the vehicle control group.

2.3. *Anti-angiogenesis Models*

2.3.1. *Corneal Angiogenesis*

Hydron pellets with or without growth factors or cells are implanted into a micropocket surgically created in the rodent cornea. Compound administration may be systemic or local (compound mixed with growth factors in the hydron pellet).

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Corneas are harvested at 7 days post implantation immediately following intracardiac infusion of colloidal carbon and are fixed in 10% formalin. Readout is qualitative scoring and/or image analysis. Qualitative scores are compared by Rank Sum test. Image analysis data is evaluated by measuring the area of neovascularization (in pixels) and group averages are compared by Student's t-test (2 tail). Significance is $p \leq 0.05$ as compared to the growth factor or cells only group.

2.3.2. *Matrigel Angiogenesis*

Matrigel, containing cells or growth factors, is injected subcutaneously. Compounds are administered p.o., i.p., i.v., i.m., or s.c. Matrigel plugs are harvested at predetermined time point(s) and prepared for readout. Readout is an ELISA-based assay for hemoglobin concentration and/or histological examination (i.e. vessel count, special staining for endothelial surface markers: CD31, factor-8). Readouts are analyzed by Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$ as compared to the vehicle control group.

3.   **Primary Antitumor Efficacy**

3.1.   *Early Therapy Models*

3.1.1. *Subcutaneous Tumor*

Tumor cells or fragments are implanted subcutaneously on Day 0. Vehicle and/or compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting

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at a time, usually on Day 1, prior to the ability to measure the tumor burden. Body weights and tumor measurements are recorded 2-3 times weekly. Mean net body and tumor weights are calculated for each data collection day. Anti-tumor efficacy may be initially determined by comparing the size of treated (T) and control (C) tumors on a given day by a Student's t-test, after the variance between groups is compared by an F-test, with significance determined at $p \leq 0.05$. The experiment may also be continued past the end of dosing in which case tumor measurements would continue to be recorded to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is $p \leq 0.05$.

3.1.2. Intraperitoneal/Intracranial Tumor Models

Tumor cells are injected intraperitoneally or intracranially on Day 0. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule starting on Day 1. Observations of morbidity and/or mortality are recorded twice daily. Body weights are measured and recorded twice weekly. Morbidity/mortality data is expressed in terms of the median time of survival and the number of long-term survivors is indicated separately. Survival times are used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment.

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3.2.   *Established Disease Model*

Tumor cells or fragments are implanted subcutaneously and grown to the desired size for treatment to begin. Once at the predetermined size range, mice are randomized into treatment groups. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value ≤ 0.05 compared to the vehicle control group.

3.3.   **Orthotopic Disease Models**

3.3.1. *Mammary Fat Pad Assay*

Tumor cells or fragments, of mammary adenocarcinoma origin, are implanted directly into a surgically exposed and reflected mammary fat pad in rodents. The fat pad is placed back in its original position and the surgical site is closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o.,

i.p., i.v., i.m., or s.c. according to a predetermined schedule. Tumor and body weights are measured and recorded 2-3 times weekly. Mean tumor weights of all groups over days post inoculation are graphed for comparison. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group.

Tumor measurements may be recorded after dosing has stopped to monitor tumor growth delay. Tumor growth delays are expressed as the difference in the median time for the treated and control groups to attain a predetermined size divided by the median time for the control group to attain that size. Growth delays are compared by generating Kaplan-Meier curves from the times for individual tumors to attain the evaluation size. Significance is p value≤ 0.05 compared to the vehicle control group. In addition, this model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ, or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

3.3.2. Intraprostatic Assay

Tumor cells or fragments, of prostatic adenocarcinoma origin, are implanted directly into a surgically exposed dorsal lobe of the prostate in rodents. The prostate is externalized through an

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abdominal incision so that the tumor can be implanted specifically in the dorsal lobe while verifying that the implant does not enter the seminal vesicles. The successfully inoculated prostate is replaced in the abdomen and the

5 incisions through the abdomen and skin are closed. Hormones may also be administered to the rodents to support the growth of the tumors. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a

10 predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal

15 followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at

20 termination of the study by counting the number of visible foci per target organ (i.e., the lungs), or measuring the target organ weight (i.e., the regional lymph nodes). The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to

25 the control group in the experiment.

3.3.3. Intrabronchial Assay

Tumor cells of pulmonary origin may be implanted intra-

30 bronchially by making an incision through the skin and exposing the trachea. The trachea is pierced with the beveled

end of a 25 gauge needle and the tumor cells are inoculated into the main bronchus using a flat-ended 27 gauge needle with a 90° bend. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the contralateral lung), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

3.3.4. Intracecal Assay

Tumor cells of gastrointestinal origin may be implanted intra-cecally by making an abdominal incision through the skin and externalizing the intestine. Tumor cells are inoculated into the cecal wall without penetrating the lumen of the intestine using a 27 or 30 gauge needle. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Body weights are measured and recorded 2-3 times weekly. At a predetermined time, the experiment is terminated and the

animal is dissected. The size of the primary tumor is measured in three dimensions using either a caliper or an ocular micrometer attached to a dissecting scope. An F-test is preformed to determine if the variance is equal or unequal followed by a Student's t-test to compare tumor sizes in the treated and control groups at the end of treatment. Significance is $p \leq 0.05$ as compared to the control group. This model provides an opportunity to increase the rate of spontaneous metastasis of this type of tumor. Metastasis can be assessed at termination of the study by counting the number of visible foci per target organ (i.e., the liver), or measuring the target organ weight. The means of these endpoints are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment.

4.   Secondary (Metastatic) Antitumor Efficacy

4.1.   *Spontaneous Metastasis*

Tumor cells are inoculated s.c. and the tumors allowed to grow to a predetermined range for spontaneous metastasis studies to the lung or liver. These primary tumors are then excised. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule which may include the period leading up to the excision of the primary tumor to evaluate therapies directed at inhibiting the early stages of tumor metastasis. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined.

Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance determined at $p \leq 0.05$ compared to the control group in the experiment for both of these endpoints.

4.2.   Forced Metastasis

Tumor cells are injected into the tail vein, portal vein, or the left ventricle of the heart in experimental (forced) lung, liver, and bone metastasis studies, respectively. Compounds are administered p.o., i.p., i.v., i.m., or s.c. according to a predetermined schedule. Observations of morbidity and/or mortality are recorded daily. Body weights are measured and recorded twice weekly. Potential endpoints include survival time, numbers of visible foci per target organ, or target organ weight. When survival time is used as the endpoint the other values are not determined. Survival data is used to generate Kaplan-Meier curves. Significance is $p \leq 0.05$ by a log-rank test compared to the control group in the experiment. The mean number of visible tumor foci, as determined under a dissecting microscope, and the mean target organ weights are compared by Student's t-test after conducting an F-test, with significance at $p \leq 0.05$ compared to the vehicle control group in the experiment for both endpoints.

EXAMPLE 11

In vivo testing of compounds/target validation

5   1.   **Pain**

Acute Pain

Acute pain is measured on a hot plate mainly in rats. Two variants of hot plate
10   testing are used: In the classical variant animals are put on a hot surface (52 to
56   C) and the latency time is measured until the animals show nocifensive
behavior, such as stepping or foot licking. The other variant is an increasing
temperature hot plate where the experimental animals are put on a surface of
neutral temperature. Subsequently this surface is slowly but constantly heated
15   until the animals begin to lick a hind paw. The temperature which is reached
when hind paw licking begins is a measure for pain threshold.

Compounds are tested against a vehicle treated control group. Substance
application is performed at different time points via different application
20   routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain
testing.

Persistent Pain

25   Persistent pain is measured with the formalin or capsaicin test, mainly in rats.
A solution of 1 to 5% formalin or 10 to 100 μg capsaicin is injected into one
hind paw of the experimental animal. After formalin or capsaicin application
the animals show nocifensive reactions like flinching, licking and biting of
the affected paw. The number of nocifensive reactions within a time frame of
30   up to 90 minutes is a measure for intensity of pain.

Compounds are tested against a vehicle treated control group. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to formalin or capsaicin administration.

Neuropathic Pain

Neuropathic pain is induced by different variants of unilateral sciatic nerve injury mainly in rats. The operation is performed under anesthesia. The first variant of sciatic nerve injury is produced by placing loosely constrictive ligatures around the common sciatic nerve. The second variant is the tight ligation of about the half of the diameter of the common sciatic nerve. In the next variant, a group of models is used in which tight ligations or transections are made of either the L5 and L6 spinal nerves, or the L% spinal nerve only. The fourth variant involves an axotomy of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact whereas the last variant comprises the axotomy of only the tibial branch leaving the sural and common nerves uninjured. Control animals are treated with a sham operation.

Postoperatively, the nerve injured animals develop a chronic mechanical allodynia, cold allodynioa, as well as a thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA; Electronic von Frey System, Somedic Sales AB, Hörby, Sweden). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy), or by means of a cold plate of 5 to 10 µC where the nocifensive reactions of the affected hind paw are counted as a measure of pain intensity. A further test for cold induced pain is the counting of nocifensive reactions, or duration of nocifensive responses after plantar administration of acetone to the affected hind limb. Chronic pain in general is

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assessed by registering the circadanian rhythms in activity (Surjo and Arndt, Universität zu Köln, Cologne, Germany), and by scoring differences in gait (foot print patterns; FOOTPRINTS program, Klapdor et al., 1997. A low cost method to analyze footprint patterns. J. Neurosci. Methods 75, 49-54).

5

Compounds are tested against sham operated and vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

10

Inflammatory Pain

Inflammatory pain is induced mainly in rats by injection of 0.75 mg carrageenan or complete Freund's adjuvant into one hind paw. The animals

15   develop an edema with mechanical allodynia as well as thermal hyperalgesia. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA). Thermal hyperalgesia is measured by means of a radiant heat source (Plantar Test, Ugo Basile, Comerio, Italy, Paw thermal

20   stimulator, G. Ozaki, University of California, USA). For edema measurement two methods are being used. In the first method, the animals are sacrificed and the affected hindpaws sectioned and weighed. The second method comprises differences in paw volume by measuring water displacement in a plethysmometer (Ugo Basile, Comerio, Italy).

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Compounds are tested against uninflamed as well as vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

30

Diabetic Neuropathic Pain

Rats treated with a single intraperitoneal injection of 50 to 80 mg/kg streptozotocin develop a profound hyperglycemia and mechanical allodynia within 1 to 3 weeks. Mechanical allodynia is measured by means of a pressure transducer (electronic von Frey Anesthesiometer, IITC Inc.-Life Science Instruments, Woodland Hills, SA, USA).

Compounds are tested against diabetic and non-diabetic vehicle treated control groups. Substance application is performed at different time points via different application routes (i.v., i.p., p.o., i.t., i.c.v., s.c., intradermal, transdermal) prior to pain testing.

2.   Parkinson's disease

6-Hydroxydopamine (6-OH-DA) Lesion

Degeneration of the dopaminergic nigrostriatal and striatopallidal pathways is the central pathological event in Parkinson's disease. This disorder has been mimicked experimentally in rats using single/sequential unilateral stereotaxic injections of 6-OH-DA into the medium forebrain bundle (MFB).

Male Wistar rats (Harlan Winkelmann, Germany), weighing 200±250 g at the beginning of the experiment, are used. The rats are maintained in a temperature- and humidity-controlled environment under a 12 h light/dark cycle with free access to food and water when not in experimental sessions. The following in vivo protocols are approved by the governmental authorities. All efforts are made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Animals are administered pargyline on the day of surgery (Sigma, St. Louis, MO, USA; 50 mg/kg i.p.) in order to inhibit metabolism of 6-OHDA by monoamine oxidase and desmethylimipramine HCl (Sigma; 25 mg/kg i.p.) in order to prevent uptake of 6-OHDA by noradrenergic terminals. Thirty minutes later the rats are anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic frame. In order to lesion the DA nigrostriatal pathway 4 μl of 0.01% ascorbic acid-saline containing 8 μg of 6-OHDA HBr (Sigma) are injected into the left medial fore-brain bundle at a rate of 1 μl/min (2.4 mm anterior, 1.49 mm lateral, -2.7 mm ventral to Bregma and the skull surface). The needle is left in place an additional 5 min to allow diffusion to occur.

Stepping Test

Forelimb akinesia is assessed three weeks following lesion placement using a modified stepping test protocol. In brief, the animals are held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. One paw is touching the table, and is then moved slowly sideways (5 s for 1 m), first in the forehand and then in the backhand direction. The number of adjusting steps is counted for both paws in the backhand and forehand direction of movement. The sequence of testing is right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test is repeated three times on three consecutive days, after an initial training period of three days prior to the first testing. Forehand adjusted stepping reveals no consistent differences between lesioned and healthy control animals. Analysis is therefore restricted to backhand adjusted stepping.

Balance Test

Balance adjustments following postural challenge are also measured during the stepping test sessions. The rats are held in the same position as described in the stepping test and, instead of being moved sideways, tilted by the experimenter towards the side of the paw touching the table. This maneuver results in loss of balance and the ability of the rats to regain balance by forelimb movements is scored on a scale ranging from 0 to 3. Score 0 is given for a normal forelimb placement. When the forelimb movement is delayed but recovery of postural balance detected, score 1 is given. Score 2 represents a clear, yet insufficient, forelimb reaction, as evidenced by muscle contraction, but lack of success in recovering balance, and score 3 is given for no reaction of movement. The test is repeated three times a day on each side for three consecutive days after an initial training period of three days prior to the first testing.

Staircase Test (Paw Reaching)

A modified version of the staircase test is used for evaluation of paw reaching behavior three weeks following primary and secondary lesion placement. Plexiglass test boxes with a central platform and a removable staircase on each side are used. The apparatus is designed such that only the paw on the same side at each staircase can be used, thus providing a measure of independent forelimb use. For each test the animals are left in the test boxes for 15 min. The double staircase is filled with 7 x 3 chow pellets (Precision food pellets, formula: P, purified rodent diet, size 45 mg; Sandown Scientific) on each side. After each test the number of pellets eaten (successfully retrieved pellets) and the number of pellets taken (touched but dropped) for each paw and the success rate (pellets eaten/pellets taken) are counted separately. After three days of food deprivation (12 g per animal per day) the

animals are tested for 11 days. Full analysis is conducted only for the last five days.

MPTP treatment

5

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) causes degeneration of mesencephalic dopaminergic (DAergic) neurons in rodents, non-human primates, and humans and, in so doing, reproduces many of the symptoms of Parkinson's disease. MPTP leads to a marked decrease in

10 the levels of dopamine and its metabolites, and in the number of dopaminergic terminals in the striatum as well as severe loss of the tyrosine hydroxylase (TH)-immunoreactive cell bodies in the substantia nigra, pars compacta.

15 In order to obtain severe and long-lasting lesions, and to reduce mortality, animals receive single injections of MPTP, and are then tested for severity of lesion 7–10 days later. Successive MPTP injections are administered on days 1, 2 and 3. Animals receive application of 4 mg/kg MPTP hydrochloride (Sigma) in saline once daily. All injections are intraperitoneal (i.p.) and the

20 MPTP stock solution is frozen between injections. Animals are decapitated on day 11.

Immunohistology

25 At the completion of behavioral experiments, all animals are anaesthetized with 3 ml thiopental (1 g/40 ml i.p., Tyrol Pharma). The mice are perfused transcardially with 0.01 M PBS (pH 7.4) for 2 min, followed by 4% paraformaldehyde (Merck) in PBS for 15 min. The brains are removed and placed in 4% paraformaldehyde for 24 h at 4°C. For dehydration they are then

30 transferred to a 20% sucrose (Merck) solution in 0.1 M PBS at 4°C until they sink. The brains are frozen in methylbutan at -20°C for 2 min and stored at

-70°C. Using a sledge microtome (mod. 3800-Frigocut, Leica), 25 μm sections are taken from the genu of the corpus callosum (AP 1.7 mm) to the hippocampus (AP 21.8 mm) and from AP 24.16 to AP 26.72. Forty-six sections are cut and stored in assorters in 0.25 M Tris buffer (pH 7.4) for immunohistochemistry.

A series of sections is processed for free-floating tyrosine hydroxylase (TH) immunohistochemistry. Following three rinses in 0.1 M PBS, endogenous peroxidase activity is quenched for 10 min in 0.3% H_2O_2 ±PBS. After rinsing in PBS, sections are preincubated in 10% normal bovine serum (Sigma) for 5 min as blocking agent and transferred to either primary anti-rat TH rabbit antiserum (dilution 1:2000).

Following overnight incubation at room temperature, sections for TH immunoreactivity are rinsed in PBS (2 x10 min) and incubated in biotinylated anti-rabbit immunoglobulin G raised in goat (dilution 1:200) (Vector) for 90 min, rinsed repeatedly and transferred to Vectastain ABC (Vector) solution for 1 h. 3,.3' -Diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.1 M PBS, supplemented with 0.005% H_2O_2 , serves as chromogen in the subsequent visualization reaction. Sections are mounted on to gelatin-coated slides, left to dry overnight, counter-stained with hematoxylin dehydrated in ascending alcohol concentrations and cleared in butylacetate. Coverslips are mounted on entellan.

Rotarod Test

We use a modification of the procedure described by Rozas and Labandeira-Garcia (1997), with a CR-1 Rotamex system (Columbus Instruments, Columbus, OH) comprising an IBM-compatible personal computer, a CIO-24 data acquisition card, a control unit, and a four-lane rotarod unit. The rotarod unit consists of a rotating spindle (diameter 7.3 cm) and individual compart-

ments for each mouse. The system software allows preprogramming of session protocols with varying rotational speeds (0–80 rpm). Infrared beams are used to detect when a mouse has fallen onto the base grid beneath the rotarod. The system logs the fall as the end of the experiment for that mouse, and the total time on the rotarod, as well as the time of the fall and all the set-up parameters, are recorded. The system also allows a weak current to be passed through the base grid, to aid training.

3. Dementia

The object recognition task

The object recognition task has been designed to assess the effects of experimental manipulations on the cognitive performance of rodents. A rat is placed in an open field, in which two identical objects are present. The rats inspects both objects during the first trial of the object recognition task. In a second trial, after a retention interval of for example 24 hours, one of the two objects used in the first trial, the 'familiar' object, and a novel object are placed in the open field. The inspection time at each of the objects is registered. The basic measures in the OR task is the time spent by a rat exploring the two object the second trial. Good retention is reflected by higher exploration times towards the novel than the 'familiar' object.

Administration of the putative cognition enhancer prior to the first trial predominantly allows assessment of the effects on acquisition, and eventually on consolidation processes. Administration of the testing compound after the first trial allows to assess the effects on consolidation processes, whereas administration before the second trial allows to measure effects on retrieval processes.

The passive avoidance task

The passive avoidance task assesses memory performance in rats and mice. The inhibitory avoidance apparatus consists of a two-compartment box with a light compartment and a dark compartment. The two compartments are separated by a guillotine door that can be operated by the experimenter. A threshold of 2 cm separates the two compartments when the guillotine door is raised. When the door is open, the illumination in the dark compartment is about 2 lux. The light intensity is about 500 lux at the center of the floor of the light compartment.

Two habituation sessions, one shock session, and a retention session are given, separated by inter-session intervals of 24 hours. In the habituation sessions and the retention session the rat is allowed to explore the apparatus for 300 sec. The rat is placed in the light compartment, facing the wall opposite to the guillotine door. After an accommodation period of 15 sec. the guillotine door is opened so that all parts of the apparatus can be visited freely. Rats normally avoid brightly lit areas and will enter the dark compartment within a few seconds.

In the shock session the guillotine door between the compartments is lowered as soon as the rat has entered the dark compartment with its four paws, and a scrambled 1 mA footshock is administered for 2 sec. The rat is removed from the apparatus and put back into its home cage. The procedure during the retention session is identical to that of the habituation sessions.

The step-through latency, that is the first latency of entering the dark compartment (in sec.) during the retention session is an index of the memory performance of the animal; the longer the latency to enter the dark compartment, the better the retention is. A testing compound in given half an hour before the shock session, together with 1 mg*kg^{-1} scopolamine.

Scopolamine impairs the memory performance during the retention session 24 hours later. If the test compound increases the enter latency compared with the scopolamine-treated controls, is likely to possess cognition enhancing potential.

The Morris water escape task

The Morris water escape task measures spatial orientation learning in rodents. It is a test system that has extensively been used to investigate the effects of putative therapeutic on the cognitive functions of rats and mice. The performance of an animal is assessed in a circular water tank with an escape platform that is submerged about 1 cm below the surface of the water. The escape platform is not visible for an animal swimming in the water tank. Abundant extra-maze cues are provided by the furniture in the room, including desks, computer equipment, a second water tank, the presence of the experimenter, and by a radio on a shelf that is playing softly.

The animals receive four trials during five daily acquisition sessions. A trial is started by placing an animal into the pool, facing the wall of the tank. Each of four starting positions in the quadrants north, east, south, and west is used once in a series of four trials; their order is randomized. The escape platform is always in the same position. A trial is terminated as soon as the animal had climbs onto the escape platform or when 90 seconds have elapsed, whichever event occurs first. The animal is allowed to stay on the platform for 30 seconds. Then it is taken from the platform and the next trial is started. If an animal did not find the platform within 90 seconds it is put on the platform by the experimenter and is allowed to stay there for 30 seconds. After the fourth trial of the fifth daily session, an additional trial is given as a probe trial: the platform is removed, and the time the animal spends in the four quadrants is measured for 30 or 60 seconds. In the probe trial, all animals start from the

same start position, opposite to the quadrant where the escape platform had been positioned during acquisition.

Four different measures are taken to evaluate the performance of an animal during acquisition training: escape latency, traveled distance, distance to platform, and swimming speed. The following measures are evaluated for the probe trial: time (s) in quadrants and traveled distance (cm) in the four quadrants. The probe trial provides additional information about how well an animal learned the position of the escape platform. If an animal spends more time and swims a longer distance in the quadrant where the platform had been positioned during the acquisition sessions than in any other quadrant, one concludes that the platform position has been learned well.

In order to assess the effects of putative cognition enhancing compounds, rats or mice with specific brain lesions which impair cognitive functions, or animals treated with compounds such as scopolamine or MK-801, which interfere with normal learning, or aged animals which suffer from cognitive deficits, are used.

The T-maze spontaneous alternation task

The T-maze spontaneous alternation task (TeMCAT) assesses the spatial memory performance in mice. The start arm and the two goal arms of the T-maze are provided with guillotine doors which can be operated manually by the experimenter. A mouse is put into the start arm at the beginning of training. The guillotine door is closed. In the first trial, the 'forced trial', either the left or right goal arm is blocked by lowering the guillotine door. After the mouse has been released from the start arm, it will negotiate the maze, eventually enter the open goal arm, and return to the start position, where it will be confined for 5 seconds, by lowering the guillotine door. Then, the animal can choose freely between the left and right goal arm (all

guillotine-doors opened) during 14 'free choice' trials. As soon a the mouse has entered one goal arm, the other one is closed. The mouse eventually returns to the start arm and is free to visit whichever go alarm it wants after having been confined to the start arm for 5 seconds. After completion of 14 free choice trials in one session, the animal is removed from the maze. During training, the animal is never handled.

The percent alternations out of 14 trials is calculated. This percentage and the total time needed to complete the first forced trial and the subsequent 14 free choice trials (in s) is analyzed. Cognitive deficits are usually induced by an injection of scopolamine, 30 min before the start of the training session. Scopolamine reduced the per-cent alternations to chance level, or below. A cognition enhancer, which is always administered before the training session, will at least partially, antagonize the scopolamine-induced reduction in the spontaneous alternation rate.

CLAIMS

1.   An isolated polynucleotide encoding a phospholipase-like enzyme poly-
peptide and being selected from the group consisting of:

   a)   a polynucleotide encoding a phospholipase-like enzyme polypeptide
          comprising an amino acid sequence selected form the group consisting
          of:

          amino acid sequences which are at least about 77% identical to
          the amino acid sequence shown in SEQ ID NO: 2; and
          the amino acid sequence shown in SEQ ID NO: 2;

   b)   a polynucleotide comprising the sequence of SEQ ID NO: 1;

   c)   a polynucleotide which hybridizes under stringent conditions to a
          polynucleotide specified in (a) and (b);

   d)   a polynucleotide the sequence of which deviates from the poly-
          nucleotide sequences specified in (a) to (c) due to the degeneration of
          the genetic code; and

   e)   a polynucleotide which represents a fragment, derivative or allelic
          variation of a polynucleotide sequence specified in (a to (d).

2.   An expression vector containing any polynucleotide of claim 1.

3.   A host cell containing the expression vector of claim 2.

4.   A substantially purified phospholipase-like enzyme polypeptide encoded by a
polynucleotide of claim 1.

5.   A method for producing a phospholipase-like enzyme polypeptide, wherein the method comprises the following steps:

a)   culturing the host cell of claim 3 under conditions suitable for the expression of the phospholipase-like enzyme polypeptide; and

b)   recovering the phospholipase-like enzyme polypeptide from the host cell culture.

6.   A method for detection of a polynucleotide encoding a phospholipase-like enzyme polypeptide in a biological sample comprising the following steps:

a)   hybridizing any polynucleotide of claim 1 to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and

b)   detecting said hybridization complex.

7.   The method of claim 6, wherein before hybridization, the nucleic acid material of the biological sample is amplified.

8.   A method for the detection of a polynucleotide of claim 1 or a phospholipase-like enzyme polypeptide of claim 4 comprising the steps of:

contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the phospholipase-like enzyme polypeptide.

9.   A diagnostic kit for conducting the method of any one of claims 6 to 8.

10.   A method of screening for agents which decrease the activity of a phospholipase-like enzyme, comprising the steps of:

contacting a test compound with any phospholipase-like enzyme polypeptide encoded by any polynucleotide of claim1;

5   detecting binding of the test compound to the phospholipase-like enzyme polypeptide, wherein a test compound which binds to the polypeptide is identified as a potential therapeutic agent for decreasing the activity of a phospholipase-like enzyme.

10   11.   A method of screening for agents which regulate the activity of a phospholipase-like enzyme, comprising the steps of:

contacting a test compound with a phospholipase-like enzyme polypeptide encoded by any polynucleotide of claim 1; and

15

detecting a phospholipase-like enzyme activity of the polypeptide, wherein a test compound which increases the phospholipase-like enzyme activity is identified as a potential therapeutic agent for increasing the activity of the phospholipase-like enzyme, and wherein a test compound which decreases

20   the phospholipase-like enzyme activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the phospholipase-like enzyme.

25   12.   A method of screening for agents which decrease the activity of a phospholipase-like enzyme, comprising the steps of:

contacting a test compound with any polynucleotide of claim 1 and detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent

30   for decreasing the activity of phospholipase-like enzyme.

13.   A method of reducing the activity of phospholipase-like enzyme, comprising the steps of:

        5   contacting a cell with a reagent which specifically binds to any polynucleotide of claim 1 or any phospholipase-like enzyme polypeptide of claim 4, whereby the activity of phospholipase-like enzyme is reduced.

14.   A reagent that modulates the activity of a phospholipase-like enzyme polypeptide or a polynucleotide wherein said reagent is identified by the method of any of the claim 10 to 12.

15.   A pharmaceutical composition, comprising:

          the expression vector of claim 2 or the reagent of claim 14 and a pharmaceutically acceptable carrier.

16.   Use of the expression vector of claim 2 or the reagent of claim 14 to produce a medicament for modulating the activity of a phospholipase-like enzyme in a disease.

17.   Use of claim 16 wherein the disease is cancer, inflammation, CNS disorder, cardiovascular disorder, chronic obstructive pulmonary disease, diabetes, or obesity.

18.   A cDNA encoding a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2.

19.   The cDNA of claim 18 which comprises SEQ ID NO: 1.

20.   The cDNA of claim 18 which consists of SEQ ID NO: 1.

21.   An expression vector comprising a polynucleotide which encodes a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2.

22.   The expression vector of claim 21 wherein the polynucleotide consists of SEQ ID NO: 1.

23.   A host cell comprising an expression vector which encodes a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2.

24.   The host cell of claim 23 wherein the polynucleotide consists of SEQ ID NO: 1.

25.   A purified polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2.

26.   The purified polypeptide of claim 25 which consists of the amino acid sequence shown in SEQ ID NO: 2.

27.   A fusion protein comprising a polypeptide having the amino acid sequence shown in SEQ ID NO: 2.

28.   A method of producing a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2, comprising the steps of:

culturing a host cell comprising an expression vector which encodes the polypeptide under conditions whereby the polypeptide is expressed; and isolating the polypeptide.

29.   The method of claim 28 wherein the expression vector comprises SEQ ID NO: 1.

30.   A method of detecting a coding sequence for a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2, comprising the steps of:

5   hybridizing a polynucleotide comprising 11 contiguous nucleotides of SEQ ID NO: 1 to nucleic acid material of a biological sample, thereby forming a hybridization complex; and

           detecting the hybridization complex.

10   31.   The method of claim 30 further comprising the step of amplifying the nucleic acid material before the step of hybridizing.

32.   A kit for detecting a coding sequence for a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2, comprising:

15
           a polynucleotide comprising 11 contiguous nucleotides of SEQ ID NO: 1; and instructions for the method of claim 30.

33.   A method of detecting a polypeptide comprising the amino acid sequence
20   shown in SEQ ID NO: 2, comprising the steps of:

           contacting a biological sample with a reagent that specifically binds to the polypeptide to form a reagent-polypeptide complex; and
           detecting the reagent-polypeptide complex.

25
34.   The method of claim 33 wherein the reagent is an antibody.

35.   A kit for detecting a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 2, comprising:

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           an antibody which specifically binds to the polypeptide; and

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instructions for the method of claim 33.

36.   A method of screening for agents which can modulate the activity of a human phospholipase-like enzyme, comprising the steps of:

contacting a test compound with a polypeptide comprising an amino acid sequence selected from the group consisting of: (1) amino acid sequences which are at least about 77% identical to the amino acid sequence shown in SEQ ID NO: 2 and (2) the amino acid sequence shown in SEQ ID NO: 2; and

detecting binding of the test compound to the polypeptide, wherein a test compound which binds to the polypeptide is identified as a potential agent for regulating activity of the human phospholipase-like enzyme.

37.   The method of claim 36 wherein the step of contacting is in a cell.

38.   The method of claim 36 wherein the cell is *in vitro*.

39.   The method of claim 36 wherein the step of contacting is in a cell-free system.

40.   The method of claim 36 wherein the polypeptide comprises a detectable label.

41.   The method of claim 36 wherein the test compound comprises a detectable label.

42.   The method of claim 36 wherein the test compound displaces a labeled ligand which is bound to the polypeptide.

43.   The method of claim 36 wherein the polypeptide is bound to a solid support.

44.   The method of claim 36 wherein the test compound is bound to a solid support.

45.   A method of screening for agents which modulate an activity of a human phospholipase-like enzyme, comprising the steps of:

contacting a test compound with a polypeptide comprising an amino acid sequence selected from the group consisting of: (1) amino acid sequences which are at least about 77% identical to the amino acid sequence shown in SEQ ID NO: 2 and (2) the amino acid sequence shown in SEQ ID NO: 2; and

detecting an activity of the polypeptide, wherein a test compound which increases the activity of the polypeptide is identified as a potential agent for increasing the activity of the human phospholipase-like enzyme, and wherein a test compound which decreases the activity of the polypeptide is identified as a potential agent for decreasing the activity of the human phospholipase-like enzyme.

46.   The method of claim 45 wherein the step of contacting is in a cell.

47.   The method of claim 45 wherein the cell is *in vitro*.

48.   The method of claim 45 wherein the step of contacting is in a cell-free system.

49.   A method of screening for agents which modulate an activity of a human phospholipase-like enzyme, comprising the steps of:

contacting a test compound with a product encoded by a polynucleotide which comprises the nucleotide sequence shown in SEQ ID NO: 1; and

detecting binding of the test compound to the product, wherein a test compound which binds to the product is identified as a potential agent for regulating the activity of the human phospholipase-like enzyme.

5

50.   The method of claim 49 wherein the product is a polypeptide.

51.   The method of claim 49 wherein the product is RNA.

10   52.   A method of reducing activity of a human phospholipase-like enzyme, comprising the step of:

contacting a cell with a reagent which specifically binds to a product encoded by a polynucleotide comprising the nucleotide sequence shown in SEQ ID

15   NO: 1, whereby the activity of a human phospholipase-like enzyme is reduced.

53.   The method of claim 52 wherein the product is a polypeptide.

20   54.   The method of claim 53 wherein the reagent is an antibody.

55.   The method of claim 52 wherein the product is RNA.

56.   The method of claim 55 wherein the reagent is an antisense oligonucleotide.

25

57.   The method of claim 56 wherein the reagent is a ribozyme.

58.   The method of claim 52 wherein the cell is *in vitro*.

30   59.   The method of claim 52 wherein the cell is *in vivo*.

60.   A pharmaceutical composition, comprising:

a reagent which specifically binds to a polypeptide comprising the amino acid
sequence shown in SEQ ID NO: 2; and

a pharmaceutically acceptable carrier.

61.   The pharmaceutical composition of claim 60 wherein the reagent is an
antibody.

62.   A pharmaceutical composition, comprising:

a reagent which specifically binds to a product of a polynucleotide com-
prising the nucleotide sequence shown in SEQ ID NO: 1; and
a pharmaceutically acceptable carrier.

63.   The pharmaceutical composition of claim 62 wherein the reagent is a
ribozyme.

64.   The pharmaceutical composition of claim 62 wherein the reagent is an
antisense oligonucleotide.

65.   The pharmaceutical composition of claim 62 wherein the reagent is an
antibody.

66.   A pharmaceutical composition, comprising:

an expression vector encoding a polypeptide comprising the amino acid
sequence shown in SEQ ID NO: 2; and

a pharmaceutically acceptable carrier.

67.   The pharmaceutical composition of claim 66 wherein the expression vector comprises SEQ ID NO: 1.

5   68.   A method of treating a phospholipase-like enzyme disfunction related disease, wherein the disease is selected from cancer, inflammation, CNS disorder, cardiovascular disorder, chronic obstructive pulmonary disease, diabetes, and obesity comprising the step of:

10   administering to a patient in need thereof a therapeutically effective dose of a reagent that modulates a function of a human phospholipase-like enzyme, whereby symptoms of the phospholipase-like enzyme disfunction related disease are ameliorated.

15   69.   The method of claim 68 wherein the reagent is identified by the method of claim 36.

70.   The method of claim 68 wherein the reagent is identified by the method of claim 45.

20

71.   The method of claim 68 wherein the reagent is identified by the method of claim 49.

Fig. 1

```
gaagcctggaacagcctcctggcctccagcagtacagtgagcaggagtccttcaccgtg
gtttccagcctttcttctatgagaccaccacccatctctacactcggaggaccccgactc
caggattctaccacgctggcctgcatctctgaataggatgatggagccagcaggagag
aaagatgagccattgagtgtaaaacacggaggccaatgaagtgtccctcaggagagc
ccctatctgttcagtacagaaacagcaactacctgaccagactgcagaaacccaagac
aagcttggagaaaatccatttcctatttccctgtgtctcatttcagtaagagaa
ggagcggaaatcagatgtcctgacaaagaccctccgatacggttcccacctcagttcat
aggctgaagccggctgacatcaacgtaattggagccctgggtgactctcacggcaggc
aatgggccgggtccacctgggaacgtcttgacgtcttgactcagtaccgaggcctg
tcctggagcgtcggcggagatgagaacatcgcaccgttacccctggcgaacatcctc
cgggaattcaacccttccctgaagggcttctctgttggcactggaaagaaaccagtcct
aatgccttcttaaaccaggctgtgtggcaggaggccgagctgtacctgtccaggcc
aggaggctggtggacctgatgaagaatgacacgaggatacacactttcaggagagactggaag
ataataaccctgtttataggcggcaatgacctctgtgattttctgcaatgatctggtccac
tattctcccagaacttcacagacaacattggaaaggccctgacatcctccatgctgag
gttccctgggcatttgtgaacctggttgacgtgcttgagatcgttcaacctgagggagctg
taccaggagaaaaagtctactgcccaaggatgatcctcagttctctgtcctgtgtc
ctgaagtttgatgataactcaacagaacttgcacctcatcgaattcaacaagaagttt
caggagaagaccaccaactgattgagagtgggcgatatgacacaagggaagatttact
gtggttgcagcgcgttcttgaaaacgtggacacgccaaagacctcggaggattgcct
gacaacttcttcgctcctgactgtttccacttcagcagcaagtctcactcccgagca
gccagtgctctgacaatatgcacatgtccgaccagtttttctggtcctttcaacccctg
tttgaaaacaagatcaatatcacacgtccgaccagtttttctggtcctttcaacccctg
```

Fig. 1 (continued)

```
agattctgggatttgttctatgccctagggtcatggggacctgctgccatgcaggac
agagcccttctgcccttgcaccctacctcagtgcatgccctgagacctgcagacatccaa
gttgtggctgctctggggattctgcaccgctgccaatgaattggctccaaaccagac
gacctcccgatgtcaccacacagtatcgggggactgtcatacagtgcaggagggacggc
tccctggagaatgtgaccaccttacctaatatcctcgggagtttaacagaaacctcaca
ggctacgccgtgggcacgggtgatgccaatgacacgaatgcattcctcaatcaagctgtt
cccggagcaaaggctgaggatcttatgagccaagtccaaactctgatgcagaagatgaaa
gatgatcatagagtaaatttccatgacagactgcaagagtcatcacagtgctgatcggaggc
agcgatttatgtgactactgcacagattcgaatctgtattctgcagccaactttgttcac
catctccgcaatgccttgactgctcctgcatagagggtgccagagtcctggtcaacctc
gtggacttcctgaaccccactatcatgcggcaggtgttcctgggaaacccagacaagtgc
ccagtgcagccagcgtttgtgtaactgcgttcctgaccctgcgggagaactcccaa
gagctagccaggccaggcctcagccgagcagcagcatgcgcgagctggtg
gggtcaggccgcgctatgacacgcaggaggacttctctggtgctgcagcccttcttccag
aacatccagctccctgcctgtcggatgggctccaccccagatacgtccttcttgccccagac
tgcatccaccaaatcagaaattccactcccagctgccagagccctttggaccaatatg
cttgaaccacttggaagcaaaacagagacccctgagacctgagagcagagatgccatcacc
tgtcccactcagaatgagcccttcctgagaaccctcgaatagtaactacacgtacccc
atcaagccagccattggaactgggcagtgacttcctgtgtacagagtggaaggcttcc
aatagtgttccaacctctgtccaccagctccgaccagcagacaacaactccagtgacctaccc
ctgggtgactctctgactgtgagtatggggagctcttggagcattggagggatgggaactcacac
acatcttgggaggggactctcttggagcattggagggatgggaactggggactcacac
acactgcccaacattctgaagaagttcaacccttacctcctggcttctctaccagcacc
```

Fig. 1 (continued)

```
tgggaggggacagcaggactaaatgtggcagcggaagggccagagctaggggacatgcca
gcccaggcctggggacctggtagagcggaatgaaaaacagccccgacatcaacctggagaaa
gactggaagctggtcacactcttcattggggtcaacgacttgtgtcattactgtgagaat
ccggaggcccacttggccacggaatatgttcagcacatccaacaggccctggacatcctc
tctgaggagctcccaagggcttttcgtcaacgtggtgggtcatggagctggctagcctg
taccagggccaaggcgggaaatgccatgctggctgccatgctcagaacaactgcacttgcctc
agacactcgcaaagctccctggagaagcaagaaactgaagaaagtgaactggaacctccag
catggcatctccagtttctctctactggcaccaatacacacccactgaacgtgaggactttgcggtt
gtggtgcccttctcttccaaaacacactcaccccactgaacgagagagggacactgac
ctcacctctcctccgaggactgtttcacttctcagaccgcgggcatgccgagatggcc
atcgcactctgaacaacatgctggaaccagtgggccgcaagactacctccaacaacttc
acccacagccgagccaaactcaagtgccccctcctcgagaagcccccgaggtgctctactgggct
cggaacagccgattgctgctcccagaccaggctgccggggagtcggcatcatcgggacagtggtctggagg
gtcccagtggcagcggggagtcggccttgtggtgggcatcatcgggacagtggtctggagg
tgcaggagaggtggccggaggggaagatcctccaatgagcctgcgcact
```

Fig. 2

```
EAWNSLLASS  RYSEQESFTV  VFQPFFYETT  PSLHSEDPRL  QDSTTLAWHL  WNRMMEPAGE
KDEPLSVKHG  RPMKCPSQES  PYLFSYRNSN  YLTRLQKPQD  KLGEKSIFLI  SLCVSFQVRE
GAEIRCPDKD  PSDTVPTSVH  RLKPADINVI  GALGDSLTAG  NGAGSTPGNV  LDVLTQYRGL
SWSVGGDENI  GTVTLANIL   REFNPSLKGF  SVGTGKETSP  NAFLNQAVAG  GRAEDLPVQA
RRLVDLMKND  TRIHFQEDWK  IITLFIGGND  LCDFCNDLVH  YSPQNFTDNI  GKALDILHAE
VPRAFVNLVT  VLEIVNLREL  YQEKKVYCPR  MILRSLCPCV  LKFDDNSTEL  ATLIEFNKKF
QEKTHQLIES  GRYDTREDFT  VVVQPFFENV  DMPKTSEGLP  DNSFFAPDCF  HFSSKSHSRA
ASALWNNMLE  PVGQKTTRHK  FENKINITCP  NQFFWSLSTL  RFWDLFYALR  GHGTWLPCRD
RAPSALHPTS  VHALRPADIQ  VVAALGDSLT  AGNGIGSKPD  DLPDVTTQYR  GLSYSAGGDG
SLENVTTLPN  ILREFNRNLT  GYAVGTGDAN  DTNAFLNQAV  PGAKAEDLMS  QVQTLMQKMK
DDHRVNFHED  WKVITVLIGG  SDLCDYCTDS  NLYSAANFVH  HLRNALDVLH  REVPRVLVNL
VDFLNPTIMR  QVFLGNPDKC  PVQQASVLCN  CVLTLRENSQ  ELARLEAFSR  AYRSSMRELV
GSGRYDTQED  FSVVLQPFFQ  NIQLPVLADG  LPDTSFFAPD  CIHPNQKFHS  QLARALWTNM
LEPLGSKTET  LDLRAEMPIT  CPTQNEPFLR  TPRNSNYTYP  IKPAIENWGS  DFLCTEWKAS
NSVPTSVHQL  RPADIKVVAA  LGDSLTVSMG  ARPNNSSDLP  TSWRGLSWSI  GGDGNLETHT
TLPNILKKFN  PYLLGFSTST  WEGTAGLNVA  AEGARARDMP  AQAWDLVERM  KNSPDINLEK
DWKLVTLFIG  VNDLCHYCEN  PEAHLATEYV  QHIQQALDIL  SEELPRAFVN  VVEVMELASL
YQGQGGKCAM  LAAQNNCTCL  RHSQSSLEKQ  ELKKVNWNLQ  HGISSFSYWH  QYTQREDFAV
VVQPFFQNTL  TPLNERGDTD  LTFFSEDCFH  FSDRGHAEMA  IALWNNMLEP  VGRKTTSNNF
THSRAKLKCP  SPESPYLYTL  RNSRLLPDQA  EEAPEVLYWA  VPVAAGVGLV  VGIIGTVVWR
CRRGGRREDP  PMSLRT
```

Fig. 3

```
MALWPSVFLL GLLPLLGRGA DQIQTSSGKN TLEGQLWPES LKTFPFPCDP KTLAESVPSE
SVHSLRPSDI KFVAAIGNVE TAPDSGADDL EEQDGTEKRP EQACMGVVTV LSDIIGRFSP
SALMPLCPET RLVPRGGAED LWMQATELVR SMRENPQLDF EHDWKLINVF FSNTSQCFPC
PSAQQKGLVL GGMDKLTRTL DYLQQEVPKA FVNLVDLSEL AAFSRWRQGA QLSPAAEPCR
CLRETSQLTK VLTQWSYLEA WDSLLASSKY NTQESFAVVF QPFFYESSLS ALLAEPPLQD
PTTLALSLWN RMMEPIGRKE EPFSEKERKP LRCPTQESPY LFTYRNSGQL TRVSQPQGKL
EVREGTEIRC PDKDPSDSVP TSVHRLKPAD IKVIGAMGDS LTAGNGAGSQ PGNILDVLTQ
YRGLSWSVGG DQNISTVTTL ANILREFNPS LQGFSVGTGR ETTSQAFFNQ AVAGARADGL
IPQAQRLVAL MKNDTRINFQ EDWKIITVFI GGNDLCDFCN DPVRYSPQNF TDNIGTALDI
LHAEIPRAFV NLVKVLEISK LRELYQETKV SCPRMILRSL CPCVLKFDDN STEIASLIET
IKEYQERTQQ LIDSGRYDTR DDFTVVLQPF FEKVNMPKTQ DGLPDNSFFA PDCFHFSSKA
HAHAASALWN NMLEPVGQKT THNDFEGAVN ITCPNQVWPF LSTYKNSVQG FGTWLPCRDR
SPSASPPTSV HALRPADIQV VAALGDSLTA GIGIGSKPND LSDGTTQYRG LSYSSGGDGS
LDNVTTLPNI LRQFNSNLMG FAVGTGDASG TNAFFNQAVP GAKARDLMSQ VQTLVQRMKD
DHRVNFQEDW KVITVQIGAS DLCDYCTDSN LYSAANFYDH LRDALDALHR EVPRALVNLV
DFMNPSVTRQ VFLGNPDKCP VQQASALCNC VLSPRENSYE LARLEALAQA YQSSLRELVE
SGRYDTREDF SVVLQPFFHS IQLPVLQDGR LDTSFFAPDC VHPNQKFHSQ LSRALWRNML
EPLGGKTDAL DLTAAITLTC PTQNEPFLRT FRNSDYTYPS RPAVENWGSD FLCTAWNASR
GVPNSVHELQ PGDIKVVAAL GDSLTLAMGA RPSNSSDPPM FWRGLSWSIG GDGALETHTT
LPNILKKFNP SILGFSTGTL EGTMGLNVAV QGARAQDMPA QARDLVERMR NSPEIDLEKD
WKLVTLFVGG NDLCHFCENP EGSSEGEYVQ HIQQALDVLY EELPRTFVNV VEVMELAGLH
QDQGGRCATL LAAQSHCTCF KYSQSSVEMQ ELKKVNWNLQ SGLSRLSYSH QYVQREDFAV
```

Fig. 3 (continued)

VVQPFFQNTL VPLNGRGDTD LTFFSDDCFH FSERGHAEMA IALWNMLEP VGHKTTSNNF
TYSRTKLKCP SPDSPYLYTL RNSRLLPDQA EADPTVLYWA VPVAAGAGLL IGILAMVAGR
GMRCRPREDP PLSLSTGL

Fig. 4

TAAAATAGCTTTGGACTTTATTTAAAATGTATCCATTCCAGGAGCATAGGCCTGGAAGAAG
CCCAGGCCCCAAGAAGTTGTGACTGTCCCCTGGCTTCCTATGGCACCAGGCATTGAAGC
ATGTCCTGGTGCCCGGGAGTGGCTGGGGCAGAGGGCAGTGAAGAGAGGGTGGCTATAGGGAG
TTTAGGGTGAGGACCCACCCCGGGCTTAGGGCACCAGTGCGCAGGCTCATTGGAGGA
TCTTCCCTCCGĆCACCTCTCCTGCACCTCCAGACCACTGTCCCGATGATGCCACCACA
AGGCCGACTCCCGCGTGCCCACTGGGACAGCCCAGTAGAGCACCTCGGGGGCTTCTTCAGCC
TGGTCTGGGAGCAGTCGGCTGTTCCGCAGGGTGTAGAGGTAAGGGCTCTCAGGAGAGGGG
CACTTGAGTTTGGCTCGGCTGTGGGTGAAGTTGTTGGAGGTAGTCTTGCGGCCCACTGGT
TCCAGCATGTTGTTCCAGAGTGCCATGGCCATCTCGGCCCGCATGCCCGGTCTGAGAAGTGA
AAACAGTCCTCGGAGAAGAAGGTGAGGTCAGTGTCCCCTCT

Fig. 5

GTCCCCTGTCTTGATGGGCTGCATAGAGAGGTGCCCAGAGTCCTGGTCAACCTCGTGGAC
TTCCTGAACCCCACTATCATGCGGCAGGTGTTCCTGGGAAACCAGACAAGTGCCCAGTG
CAGCAGGCCAGCGTTTGTGTAACTGCGTTCTGACCCTGCGGGAGAACTCCCAAGAGCTA
GCCAGGCTGGAGCCTTCAGCCGAGCCTACCGGAGCAGCATGCGCGAGCTGGTGGGGTCA
GGCCGCTATGACACGCAGGAGACTTCTCTGTGTGCTGCAGCCCTTCTTCCAGAACATC
CAGCTCCCTGTCCTGGCGGATGGGCTCCCAGATACGTCCTTCTTTGCCCCAGACTGCATC
CACCCAAATCAGAGAATTCCACTCCCAGCTGGCCAGAGCCCTTTGGACCAATATGCTTGAA
CCACTTGGAAGCAAAACAGAGACCCCTGAGACCCCTCCTGAGAACCCCTCGGAACCCCTGTCCC
ACTCAGAATGAGCCCTTCCTGAGAACCCCTCGGAATAAGAACTACACGTACCCCATCAAG
CCAGCCATTGAGAACTGGGGCAGTGACTTTCTGTGTACAGAAGCTTCCAATAGT
GGTCCAAT

Fig. 6

CCCAGCTGGCCAGAGCCTTTGGACCAATATGCTTGAACCACTTGGAAGCAAAACAGAGACC
CTGGACCTGAGAGCAGCAGGATGCCCATCACCTGTCCCACTCAGAATGAGCCCTTCCTGAGA
ACCCCTCGGAATAACTACACGTACCCCATCAAGCCAGCCATTGAGAACTGGGGCAGT
GACTTCCTGTGTACAGAGTGGAAGGCTTCCAATAGTGTTCCAACCTCTCGTCCACCAGCTC
CGACCAGCAGACATCAAAGTGGTGG

Fig. 7

TAAAATAGCTTTGGACTTTATTTAAAATGTATCCATTCCAGGAGCATAGGCCTGGAAGAAG
CCCAGGCCCCAAGAAGTTGTGACTGTCCCCTGGGCTTCCTAGGGCCACCAGGCATTGAAGC
ATGTCCTGGTGGCCGGGAGTGGCTGGGGCAGAGGGCGGTGAAGAGGGTGGCTATAGGGAG
TTTAGGGTGAGGACCCACCCCCGGGCCACCACAGTGCGCAGGCTCATTGGAGGA
TCTTCCCTCCGGCCACCTCTCCTGCACCTCCAGACCACTGTCCCGATGATGCCCACCACA
AGGCCGACTCCCGCTGCCACTGGGACAGCCCAGTAGAGCACCTCGGGGGCTTCTTCAGCC
TGGTCTGGGAGCAGTCGGCTGTTCCGCAGGGTGTAGAGGTAAGGGCTCTCAGGAGAGGNG
CACTTGAGTTTGGCTCGGCTGTGGGT

Fig. 8

BLASTP - alignment of 359_protein (SEQ ID NO:2) against
swiss|Q05017|PHLX_RABIT PHOSPHOLIPASE ADRAB-B PRECURSOR (EC
3.1.-.-).//::trembl|Z12841|OCPPHLIP_1

product: "Phospholipase"; O.cuniculus mRNA for phospholipase.

//::gp|Z12841|1690 product: "Phospholipase"; O.cuniculus mRNA for
phospholipase.

This hit is scoring at : 0.0 (expectation value)
Alignment length (overlap) : 1217
Identities : 77 %
Scoring matrix : BLOSUM62 (used to infer consensus pattern)
Database searched : nrdb

Q: 1 EAWNSLLASSRYSEQESFTVVFQPFFYETTPSLHSEDPRLQDSTTLAWHLWNRMMEPAGE
          EAW:SLLASS:Y:.QESF.VVFQPFFYE:: S. .:P LQD.TTLA .LWNRMMEP.G.
H: 259 EAWDSLLASSKYNTQESFAVVFQPFFYESSLSALLAEPPLQDPTTLALSLWNRMMEPIGR

KDEPLSVKHGRPMKCPSQESPYLFSYRNSNYLTRLQKPQDKLGEKSIFLISLCVSFQVRE
K:EP.S K. :P::CP:QESPYLF:YRNS.LTR::PQ.KL   :VRE
KEEPFSEKERKPLRCPTQESPYLFTYRNSGQLTRVSQPQGKL----------EVRE

Fig. 8 (continued)

prosite signature

```
GAEIRCPDKDPSDTVPTSVHRLKPADINVIGALGDSLTAGNGAGSTPGNVLDVLTQYRGL
G.EIRCPDKDPSD:VPTSVHRLKPADI.VIGA:GDSLTAGNGAGS.PGN:LDVLTQYRGL
GTEIRCPDKDPSDSVPTSVHRLKPADIKVIGAMGDSLTAGNGAGSQPGNILDVLTQYRGL

SWSVGGDENIGTVTTLANILREFNPSLKGFSVGTGKETSPNAFLNQAVAGGRAEDLPVQA
SWSVGGD:NI.TVTTLANILREFNPSL:GFSVGTG:ET:.AF.NQAVAG.RA:.L  QA
SWSVGGDQNISTVTTLANILREFNPSLQGFSVGTGRETTSQAFFNQAVAGARADGLIPQA

RRLVDLMKNDTRIHFQEDWKIITLFIGGNDLCDFCNDLVHYSPQNFTDNIGKALDILHAE
:RLV LMKNDTRI:FQEDWKIIT:FIGGNDLCDFCND V.YSPQNFTDNIG.ALDILHAE
QRLVALMKNDTRINFQEDWKIITVFIGGNDLCDFCNDPVRYSPQNFTDNIGTALDILHAE

VPRAFVNLVTVLEIVNLRELYQEKKVYCPRMILRSLCPCVLKFDDNSTELATLIEFNKKF
:PRAFVNLV.VLEI .LRELYQE.KV CPRMILRSLCPCVLKFDDNSTE:A:LIE K::
IPRAFVNLVKVLEISKLRELYQETKVSCPRMILRSLCPCVLKFDDNSTEIASLIETIKEY

QEKTHQLIESGRYDTREDFTVVQPFFENVDMPKTSEGLPDNSFFAPDCFHFSSKSHSRA
QE:T.QLI:SGRYDTR:DFTVV:QPFFE.V:MPKT.:GLPDNSFFAPDCFHFSSK:H:.A
QERTQQLIDSGRYDTRDDFTVVLQPFFEKVNMPKTQDGLPDNSFFAPDCFHFSSKAHAHA
```

Fig. 8 (continued)

```
ASALWNNMLEPVGQKTTRHKFENKINITCPNQFFWSLSTLRFWDLFYALRGHGTWLPCRD
ASALWNNMLEPVGQKTT..FE..:NITCPNQ.: LST.:      :::G.GTWLPCRD
ASALWNNMLEPVGQKTTHNDFEGAVNITCPNQVWPFLSTYK----NSVQGFGTWLPCRD

                     prosite signature

RAPSALHPTSVHALRPADIQVVAALGDSLTAGNGIGSKPDDLPDVTTQYRGLSYSAGGDG
R:PSA  PTSVHALRPADIQVVAALGDSLTAG GIGSKP:DL.D TTQYRGLSYS:GGDG
RSPSASPPTSVHALRPADIQVVAALGDSLTAGIGIGSKPNDLSDGTTQYRGLSYSSGGDG

SLENVTTLPNILREFNRNLTGYAVGTGDANDTNAFLNQAVPGAKAEDLMSQVQTLMQKMK
SL:NVTTLPNILR:FN.NL.G:AVGTGDA:.TNAF.NQAVPGAKA.DLMSQVQTL:Q:MK
SLDNVTTLPNILRQFNSNLMGFAVGTGDASGTNAFFNQAVPGAKARDLMSQVQTLVQRMK

DDHRVNFHEDWKVITVLIGGSDLCDYCTDSNLYSAANFVHHLRNALDVLHREVPRVLVNL
DDHRVNF.EDWKVITV IG.SDLCDYCTDSNLYSAANF..HLR:ALD.LHREVPR.LVNL
DDHRVNFQEDWKVITVQIGASDLCDYCTDSNLYSAANFYDHLRDALDALHREVPRALVNL

VDFLNPTIMRQVFLGNPDKCPVQQASVLCNCVLTLRENSQELARLEAFSRAYRSSMRELV
VDF:NP::.RQVFLGNPDKCPVQQAS.LCNCVL: RENS.ELARLEA.::AY:SS:RELV
VDFMNPSVTRQVFLGNPDKCPVQQASALCNCVLSPRENSYELARLEALAQAYQSSLRELV
```

Fig. 8 (continued)

```
GSGRYDTQEDFSVVLQPFFQNIQLPVLADGLPDTSFFAPDCIHPNQKFHSQLARALWTNM
SGRYDT:EDFSVVLQPFF.:IQLPVL.DG  DTSFFAPDC:HPNQKFHSQL:RALW.NM
ESGRYDTREDFSVVLQPFFHSIQLPVLQDGRLDTSFFAPDCVHPNQKFHSQLSRALWRNM

LEPLGSKTETLDLRAEMPITCPTQNEPFLRTPRNSNYTYPIKPAIENWGSDFLCTEWKAS
LEPLG.KT:.LDL.A.:.:TCPTQNEPFLRT RNS:YTYP :PA:ENWGSDFLCT.W.AS
LEPLGGKTDALDLTAAILTCPTQNEPFLRTFRNSDYTYPSRPAVENWGSDFLCTAWNAS

NSVPTSVHQLRPADIKVVAALGDSLTVSMGARPNNSSDLPTSWRGLSWSIGGDGNLETHT
..VP.SVH:L:P.DIKVVAALGDSLT::MGARP:NSSD P. WRGLSWSIGGDG LETHT
RGVPNSVHELQPGDIKVVAALGDSLTLAMGARPSNSSDPPMFWRGLSWSIGGDGALETHT

TLPNILKKFNPYLLGFSTSTWEGTAGLNVAAEGARARDMPAQAWDLVERMKNSPDINLEK
TLPNILKKFNP :LGFST.T EGT.GLNVA.:GARA:DMPAQA DLVERM:NSP:I:LEK
TLPNILKKFNPSILGFSTGTLEGTMGLNVAVQGARAQDMPAQARDLVERMRNSPEIDLEK

DWKLVTLFIGVNDLCHYCENPEAHLATEYVQHIQQALDILSEELPRAFVNVVEVMELASL
DWKLVTLF:G NDLCH:CENPE.. . EYVQHIQQALD:L EELPR.FVNVVEVMELA.L
DWKLVTLFVGGNDLCHFCENPEGSSEGEYVQHIQQALDVLYEELPRTFVNVVEVMELAGL

YQGQGGKCA-MLAAQNNCTCLRHSQSSLEKQELKKVNWNLQHGISSFSYWHQYTQREDFA
:Q.QGG:CA :LAAQ::CTC.::SQSS:E.QELKKVNWNLQ.G:S..SY HQY.QREDFA
HQDQGGRCATLLAAQSHCTCFKYSQSSVEMQELKKVNWNLQSGLSRLSYSHQYVQREDFA
```

Fig. 8 (continued)

```
VVVQPFFQNTLTPLNERGDTDLTFFSEDCFHFSDRGHAEMAIALWNNMLEPVGRKTTSNN
VVVQPFFQNTL.PLN RGDTDLTFFS:DCFHFS:RGHAEMAIALWNNMLEPVG.KTTSNN
VVVQPFFQNTLVPLNGRGDTDLTFFSDDCFHFSERGHAEMAIALWNNMLEPVGHKTTSNN

FTHSRAKLKCPSPESPYLYTLRNSRLLPDQAEEAPEVLYWAVPVAAGVGLVVGIIGTVVW
FT:SR.KLKCPSP:SPYLYTLRNSRLLPDQAE. P.VLYWAVPVAAG.GL::GI:..V.
FTYSRTKLKCPSPDSPYLYTLRNSRLLPDQAEADPTVLYWAVPVAAGAGLLIGILAMVAG

RCRRGRREDPPMSLRT    1216
R .R  REDPP:SL.T
RGMRCRPREDPPLSLST   1456
```

Ser is the active site.

Transmembrane segment:

From 1176 to 1198: length= 23,

```
1176  VLYWAVPVAA GVGLVVGIIG TVV
```

Fig. 9

Prosite search result

Access#	From->To	Name	Doc#
PS00016	1096->1099	RGD	PDOC00016
PS01098	150->161	LIPASE_GDSL_SER	PDOC00842
PS01098	502->513	LIPASE_GDSL_SER	PDOC00842

Fig. 10

HMMPFAM - alignment of 359_protein (SEQ ID NO:3) against pfam|hmm|Lipase_GDSL
Lipase/Acylhydrolase with GDSL-like m

This hit is scoring at : 168.6 Expect: 1e-46
Scoring matrix : BLOSUM62 (used to infer consensus pattern)

```
Q:  149   VIGALGDSLTAGNGAGSTPGNVLDVLTQYRGLSWSVGGDENIGTVTTLANILREFNPSLK
                : ALGDSLT G.G GS.. .: D ..QYRGLS S  GD N... T.L.:IL.:
H:   1    kvvalGDSLtdgggngsestklkdnyrqyrglspssvgdgnfsngtvlpdilakalgikl

          G------FSVGTGKETSPNAFLNQAVAGGRAEDLPVQARRLVD-------LMKNDTG
          F.VG. :E... . LN AV.G..AEDL  : .L:D        M .. :
          gVgikeksilfrvgklqengsltglNfAvgGataedllkrlpnlldqvikksqykmygks

          RIHFQEDWKIITLFIGGNDLCDFCND          277
          R . Q:D .::T: :GGNDLCD: .D
          rkksqkdpdlvtielGgnNDlcdyfrd        146
```

Fig. 11

HMMPFAM - alignment of 359_protein (SEQ ID NO:2) against
pfam|hmm|Lipase_GDSL Lipase/Acylhydrolase with GDSL-like m

This hit is scoring at : 172.2 Expect: 8.7e-48

Scoring matrix : BLOSUM62 (used to infer consensus pattern)

```
Q:  501 VVAALGDSLTAGNGIGSKPDDLPDVTTQYRGLSYSAGGDGSLENVTTLPNILREFNRNLT
            V.ALGDSLT G.G GS:..L.D  .QYRGLS S: GDG:..N T.LP:IL.:
H:    1 kvvalGDSLtdgggngsestklkdnyrqyrglspssvgdgnfsngtvlpdilakalgikl

        G-------YAVGTGDANDTNAFLNQAVPGAKAEDLMSQVQTLMQ-------KMKDDH
        G       :.VG. .N.: LN AV GA.AEDL.::..L::        KM ...
        gVgikeksilfrvgklqengsltglNfAvgGataedllkrlpnlldqvikksqykmygks

        RVNFHEDWKVITVLIGGSDLCDYCTD       629
        R. .:D .::T: :GG:DLCDY .D
        rkksqkdpdlvtielGgNDlcdyfrd      146
```

Fig. 12

HMMPFAM - alignment of 359_protein (SEQ ID NO:2) against pfam|hmm|Lipase_GDSL
Lipase/Acylhydrolase with GDSL-like m

This hit is scoring at : 155.2 Expect: 1.1e-42
Scoring matrix : BLOSUM62 (used to infer consensus pattern)

```
Q:   857 VVAALGDSLTVSMGARPNN--SSDLPTSWRGLSWSIGGDGNLETHTTLPNILKKFNPYLL
             V.ALGDSLT . G   . : .D  . :RGLS S GDGN... T.LP:IL.K  . L
H:     1 kvvalGDSLtdgggngsestklkdnyrqyrglspssvgdgnfsngtvlpdilakalgikl

     G--------FSTSTWEGTAGLNVAAEGARARDMPAQAWDLVE-------RMKNSP
     G        ....  ...GLN.A. GA.A.D: .: :L::  :M ...
     gVgikeksilfrvgklqengsltglNfAvgGataedllkrlpnlldqvikksqykmygks

     DINLEKDWKLVTLFIGVNDLCHYCEN     980
      :KD .LVT: :G NDLC.Y .:
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His Leu Trp Asn Arg Met Met Glu Pro Ala Gly Glu Lys Asp Glu Pro
        50   55   60
Leu Ser Val Lys His Gly Arg Pro Met Lys Cys Pro Ser Gln Glu Ser
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Pro Tyr Leu Phe Ser Tyr Arg Asn Ser Asn Tyr Leu Thr Arg Leu Gln
                85   90   95
Lys Pro Gln Asp Lys Leu Gly Glu Lys Ser Ile Phe Leu Ile Ser Leu
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Cys Val Ser Phe Gln Val Arg Glu Gly Ala Glu Ile Arg Cys Pro Asp
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Tyr Arg Gly Leu Ser Trp Ser Val Gly Gly Asp Glu Asn Ile Gly Thr
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Val Thr Thr Leu Ala Asn Ile Leu Arg Glu Phe Asn Pro Ser Leu Lys
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Gly Phe Ser Val Gly Thr Gly Lys Glu Thr Ser Pro Asn Ala Phe Leu
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Glu Asp Trp Lys Ile Ile Thr Leu Phe Ile Gly Gly Asn Asp Leu Cys
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Cys Pro Cys Val Leu Lys Phe Asp Asp Asn Ser Thr Glu Leu Ala Thr
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Cys Asn Cys Val Leu Thr Leu Arg Glu Asn Ser Gln Glu Leu Ala Arg
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Gly Ser Gly Arg Tyr Asp Thr Gln Glu Asp Phe Ser Val Val Leu Gln
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His Ser Gln Leu Ala Arg Ala Leu Trp Thr Asn Met Leu Glu Pro Leu
            770   775   780

Gly Ser Lys Thr Glu Thr Leu Asp Leu Arg Ala Glu Met Pro Ile Thr
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Cys Pro Thr Gln Asn Glu Pro Phe Leu Arg Thr Pro Arg Asn Ser Asn
                805   810   815

Tyr Thr Tyr Pro Ile Lys Pro Ala Ile Glu Asn Trp Gly Ser Asp Phe
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Leu Cys Thr Glu Trp Lys Ala Ser Asn Ser Val Pro Thr Ser Val His
            835   840   845

Gln Leu Arg Pro Ala Asp Ile Lys Val Val Ala Ala Leu Gly Asp Ser
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Leu Thr Val Ser Met Gly Ala Arg Pro Asn Asn Ser Ser Asp Leu Pro
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                885   890   895

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Val Ala Ala Glu Gly Ala Arg Ala Arg Asp Met Pro Ala Gln Ala Trp
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Asp Trp Lys Leu Val Thr Leu Phe Ile Gly Val Asn Asp Leu Cys His
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Tyr Cys Glu Asn Pro Glu Ala His Leu Ala Thr Glu Tyr Val Gln His
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Cys Leu Arg His Ser Gln Ser Ser Leu Glu Lys Gln Glu Leu Lys
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Lys Val Asn Trp Asn Leu Gln His Gly Ile Ser Ser Phe Ser Tyr
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    1085   1090   1095

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Arg Ala Lys Leu Lys Cys Pro Ser Pro Glu Ser Pro Tyr Leu Tyr
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Thr Leu Arg Asn Ser Arg Leu Leu Pro Asp Gln Ala Glu Glu Ala
    1160   1165   1170
Pro Glu Val Leu Tyr Trp Ala Val Pro Val Ala Ala Gly Val Gly
    1175   1180   1185
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Glu Gly Gln Leu Trp Pro Glu Ser Leu Lys Thr Phe Pro Phe Pro Cys
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Thr Ala Pro Asp Ser Gly Ala Asp Asp Leu Glu Glu Gln Asp Gly Thr
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Glu His Asp Trp Lys Leu Ile Asn Val Phe Phe Ser Asn Thr Ser Gln
            165   170   175
Cys Phe Pro Cys Pro Ser Ala Gln Gln Lys Gly Leu Val Leu Gly Gly
            180   185   190

Met Asp Lys Leu Thr Arg Thr Leu Asp Tyr Leu Gln Gln Glu Val Pro
            195   200   205

Lys Ala Phe Val Asn Leu Val Asp Leu Ser Glu Leu Ala Ala Phe Ser
            210   215   220

Arg Trp Arg Gln Gly Ala Gln Leu Ser Pro Ala Ala Glu Pro Cys Arg
225   230   235   240

Cys Leu Arg Glu Thr Ser Gln Leu Thr Lys Val Leu Thr Gln Trp Ser
                245   250   255

Tyr Leu Glu Ala Trp Asp Ser Leu Leu Ala Ser Ser Lys Tyr Asn Thr
                260   265   270

Gln Glu Ser Phe Ala Val Val Phe Gln Pro Phe Phe Tyr Glu Ser Ser
            275   280   285

Leu Ser Ala Leu Leu Ala Glu Pro Pro Leu Gln Asp Pro Thr Thr Leu
            290   295   300

Ala Leu Ser Leu Trp Asn Arg Met Met Glu Pro Ile Gly Arg Lys Glu
305   310   315   320

Glu Pro Phe Ser Glu Lys Glu Arg Lys Pro Leu Arg Cys Pro Thr Gln
                325   330   335

Glu Ser Pro Tyr Leu Phe Thr Tyr Arg Asn Ser Gly Gln Leu Thr Arg
                340   345   350

Val Ser Gln Pro Gln Gly Lys Leu Glu Val Arg Glu Gly Thr Glu Ile
            355   360   365

Arg Cys Pro Asp Lys Asp Pro Ser Asp Ser Val Pro Thr Ser Val His
            370   375   380

Arg Leu Lys Pro Ala Asp Ile Lys Val Ile Gly Ala Met Gly Asp Ser
385   390   395   400

Leu Thr Ala Gly Asn Gly Ala Gly Ser Gln Pro Gly Asn Ile Leu Asp
                405   410   415

Val Leu Thr Gln Tyr Arg Gly Leu Ser Trp Ser Val Gly Gly Asp Gln
            420   425   430

Asn Ile Ser Thr Val Thr Thr Leu Ala Asn Ile Leu Arg Glu Phe Asn
            435   440   445

Pro Ser Leu Gln Gly Phe Ser Val Gly Thr Gly Arg Glu Thr Thr Ser
            450   455   460

Gln Ala Phe Phe Asn Gln Ala Val Ala Gly Ala Arg Ala Asp Gly Leu
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Glu Ile Ala Ser Leu Ile Glu Thr Ile Lys Glu Tyr Gln Glu Arg Thr
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Gln Gln Leu Ile Asp Ser Gly Arg Tyr Asp Thr Arg Asp Asp Phe Thr
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Val Val Leu Gln Pro Phe Phe Glu Lys Val Asn Met Pro Lys Thr Gln
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Asp Gly Leu Pro Asp Asn Ser Phe Phe Ala Pro Asp Cys Phe His Phe
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Ser Ser Lys Ala His Ala His Ala Ala Ser Ala Leu Trp Asn Asn Met
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Leu Glu Pro Val Gly Gln Lys Thr Thr His Asn Asp Phe Glu Gly Ala
            675   680   685
Val Asn Ile Thr Cys Pro Asn Gln Val Trp Pro Phe Leu Ser Thr Tyr
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Ser Pro Ser Ala Ser Pro Pro Thr Ser Val His Ala Leu Arg Pro Ala
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Asp Ile Gln Val Val Ala Ala Leu Gly Asp Ser Leu Thr Ala Gly Ile
            740   745   750
Gly Ile Gly Ser Lys Pro Asn Asp Leu Ser Asp Gly Thr Thr Gln Tyr
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Arg Gly Leu Ser Tyr Ser Ser Gly Gly Asp Gly Ser Leu Asp Asn Val
        770   775   780
Thr Thr Leu Pro Asn Ile Leu Arg Gln Phe Asn Ser Asn Leu Met Gly
785   790   795   800
Phe Ala Val Gly Thr Gly Asp Ala Ser Gly Thr Asn Ala Phe Phe Asn
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Gln Ala Val Pro Gly Ala Lys Ala Arg Asp Leu Met Ser Gln Val Gln
            820   825   830
Thr Leu Val Gln Arg Met Lys Asp Asp His Arg Val Asn Phe Gln Glu
            835   840   845
Asp Trp Lys Val Ile Thr Val Gln Ile Gly Ala Ser Asp Leu Cys Asp
        850   855   860
Tyr Cys Thr Asp Ser Asn Leu Tyr Ser Ala Ala Asn Phe Tyr Asp His
865   870   875   880
Leu Arg Asp Ala Leu Asp Ala Leu His Arg Glu Val Pro Arg Ala Leu
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Val Asn Leu Val Asp Phe Met Asn Pro Ser Val Thr Arg Gln Val Phe
                900   905   910
Leu Gly Asn Pro Asp Lys Cys Pro Val Gln Gln Ala Ser Ala Leu Cys
            915   920   925

Asn Cys Val Leu Ser Pro Arg Glu Asn Ser Tyr Glu Leu Ala Arg Leu
930   935   940

Glu Ala Leu Ala Gln Ala Tyr Gln Ser Ser Leu Arg Glu Leu Val Glu
945   950   955   960

Ser Gly Arg Tyr Asp Thr Arg Glu Asp Phe Ser Val Val Leu Gln Pro
965   970   975

Phe Phe His Ser Ile Gln Leu Pro Val Leu Gln Asp Gly Arg Leu Asp
980   985   990

Thr Ser Phe Phe Ala Pro Asp Cys Val His Pro Asn Gln Lys Phe His
995   1000   1005

Ser Gln Leu Ser Arg Ala Leu Trp Arg Asn Met Leu Glu Pro Leu
1010   1015   1020

Gly Gly Lys Thr Asp Ala Leu Asp Leu Thr Ala Ala Ile Thr Leu
1025   1030   1035

Thr Cys Pro Thr Gln Asn Glu Pro Phe Leu Arg Thr Phe Arg Asn
1040   1045   1050

Ser Asp Tyr Thr Tyr Pro Ser Arg Pro Ala Val Glu Asn Trp Gly
1055   1060   1065

Ser Asp Phe Leu Cys Thr Ala Trp Asn Ala Ser Arg Gly Val Pro
1070   1075   1080

Asn Ser Val His Glu Leu Gln Pro Gly Asp Ile Lys Val Val Ala
1085   1090   1095

Ala Leu Gly Asp Ser Leu Thr Leu Ala Met Gly Ala Arg Pro Ser
1100   1105   1110

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1115   1120   1125

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1130   1135   1140

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1145   1150   1155

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1175   1180   1185

Met Arg Asn Ser Pro Glu Ile Asp Leu Glu Lys Asp Trp Lys Leu
1190   1195   1200

Val Thr Leu Phe Val Gly Gly Asn Asp Leu Cys His Phe Cys Glu
1205   1210   1215

Asn Pro Glu Gly Ser Ser Glu Gly Glu Tyr Val Gln His Ile Gln
1220   1225   1230

Gln Ala Leu Asp Val Leu Tyr Glu Glu Leu Pro Arg Thr Phe Val
1235   1240   1245

Asn Val Val Glu Val Met Glu Leu Ala Gly Leu His Gln Asp Gln
1250   1255   1260

Gly Gly Arg Cys Ala Thr Leu Leu Ala Ala Gln Ser His Cys Thr
1265   1270   1275

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Cys Phe  Lys Tyr Ser Gln Ser  Ser Val Glu Met Gln  Glu Leu Lys
    1280                 1285                 1290
Lys Val  Asn Trp Asn Leu Gln  Ser Gly Leu Ser Arg  Leu Ser Tyr
    1295                 1300                 1305
Ser His  Gln Tyr Val Gln Arg  Glu Asp Phe Ala Val  Val Val Gln
    1310                 1315                 1320
Pro Phe  Phe Gln Asn Thr Leu  Val Pro Leu Asn Gly  Arg Gly Asp
    1325                 1330                 1335
Thr Asp  Leu Thr Phe Phe Ser  Asp Asp Cys Phe His  Phe Ser Glu
    1340                 1345                 1350
Arg Gly  His Ala Glu Met Ala  Ile Ala Leu Trp Asn  Asn Met Leu
    1355                 1360                 1365
Glu Pro  Val Gly His Lys Thr  Thr Ser Asn Asn Phe  Thr Tyr Ser
    1370                 1375                 1380
Arg Thr  Lys Leu Lys Cys Pro  Ser Pro Asp Ser Pro  Tyr Leu Tyr
    1385                 1390                 1395
Thr Leu  Arg Asn Ser Arg Leu  Leu Pro Asp Gln Ala  Glu Ala Asp
    1400                 1405                 1410
Pro Thr  Val Leu Tyr Trp Ala  Val Pro Val Ala Ala  Gly Ala Gly
    1415                 1420                 1425
Leu Leu  Ile Gly Ile Leu Ala  Met Val Ala Gly Arg  Gly Met Arg
    1430                 1435                 1440
Cys Arg  Pro Arg Glu Asp Pro  Pro Leu Ser Leu Ser  Thr Gly Leu
    1445                 1450                 1455
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<210> 4
<211> 583
<212> DNA
<213> Homo sapiens

<400> 4

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atgtcctggt ggccgggagt ggctggggca gagggcagtg aagagggtgg ctatagggag   180
tttagggtga ggacccaccc ccgggcctag agggcaccag tgcgcaggct cattggagga   240
tcttccctcc ggccacctct cctgcacctc cagaccactg tcccgatgat gcccaccaca   300
aggccgactc ccgctgccac tgggacagcc cagtagagca cctcgggggc ttcttcagcc   360
tggtctggga gcagtcggct gttccgcagg gtgtagaggt aagggctctc aggagagggg   420
cacttgagtt tggctcggct gtgggtgaag ttgttggagg tagtcttgcg gcccactggt   480
tccagcatgt tgttccagag tgcgatggcc atctcggcat gcccgcggtc tgagaagtga   540
aaacagtcct cggagaagaa ggtgaggtca gtgtcccctc tct                     583
```

<210> 5
<211> 608
<212> DNA
<213> Homo sapiens

<400> 5

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ttcctgaacc ccactatcat gcggcaggtg ttcctgggaa acccagacaa gtgcccagtg   120
cagcaggcca gcgttttgtg taactgcgtt ctgaccctgc gggagaactc ccaagagcta   180
gccaggctgg aggccttcag ccgagcctac cggagcagca tgcgcgagct ggtggggtca   240
ggccgctatg acacgcagga ggacttctct gtggtgctgc agcccttctt ccagaacatc   300
cagctccctg tcctggcgga tgggctccca gatacgtcct tctttgcccc agactgcatc   360
cacccaaatc agaaattcca ctcccagctg gccagagccc tttggaccaa tatgcttgaa   420
ccacttggaa gcaaaacaga gaccctggac ctgagagcag agatgcccat cacctgtccc   480
actcagaatg agcccttcct gagaacccct cggaataaga actacacgta ccccatcaag   540
ccagccattg agaactgggg cagtgacttt ctgtgtacag aagtggaagc ttccaatagt   600
ggtccaat                                                             608
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<210> 6
<211> 265
<212> DNA
<213> Homo sapiens

<400> 6

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cccagctggc agagcctttg gaccaatatg cttgaaccac ttggaagcaa aacagagacc    60
ctggacctga gagcagagat gcccatcacc tgtcccactc agaatgagcc cttcctgaga   120
acccctcgga atagtaacta cacgtacccc atcaagccag ccattgagaa ctggggcagt   180
gacttcctgt gtacagagtg gaaggcttcc aatagtgttc caacctctgt ccaccagctc   240
cgaccagcag acatcaaagt ggtgg                                         265
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<210> 7
<211> 446
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> (419)..(419)
<223> n = a,t,g, or c

<400> 7

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cccaggcccc aagaagttgt gactgtcccc tgggcttcct agggcaccag gcattgaagc   120
atgtcctggt ggccgggagt ggctggggca gagggcggtg aagagggtgg ctataggag    180
tttagggtga ggacccaccc ccgggcctag agggccacag tgcgcaggct cattggagga   240
tcttccctcc ggccacctct cctgcacctc cagaccactg tcccgatgat gcccaccaca   300
aggccgactc ccgctgccac tgggacagcc cagtagagca cctcggg ggc ttcttcagcc   360
tggtctggga gcagtcggct gttccgcagg gtgtagaggt aagggctctc aggagaggng   420
cacttgagtt tggctcggct gtgggt                                        446
```